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Physiological Impact of Lipoxin A4 on Cystic Fibrosis Bronchial Epithelium

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Physiological Impact of Lipoxin A_4 on Cystic Fibrosis Bronchial Epithelium

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by

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed Gerard Higgins

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Date 1-05-14.

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"You can't connect the dots looking forward; you can only connect them looking backwards; trust that the dots will somehow connect in your future" Steve Jobs

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Abstract

This thesis reports a novel role of lipoxin A_4 (LXA_4) in mediating physiological effects within the airways of cystic fibrosis (CF) patients. CF is caused by a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This mutation leads to a dysfunctional chloride transport system resulting in dehydration of the airway surface liquid (ASL) layer, impaired mucociliary clearance, lung infection and inflammation. The eicosanoid LXA_4 described as a signal of the resolution of inflammation and produced at the site of inflammation has been reported to be decreased in the lungs of patients with CF. The decrease in LXA_4 observed in CF lungs could participate to chronic airway inflammation in this disease. Using live cell imaging, we investigated the role of LXA_4 on ASL height in non-CF and CF bronchial epithelial cells cultured in air/liquid interface. We also examined the effects of TA39, LXA_4 stable analogue, in mediating ASL height. LXA_4 and TA39 increase the ASL height in a dose and time dependent manner in both the non-CF and CF airway epithelium. The mechanism behind the increase in ASL height was also investigated and we found that LXA_4 stimulated an apical adenosine triphosphate (ATP) release in bronchial epithelial cells to activate a purinoreceptor pathway, in particular the P2Y11 pathway, which increased intracellular calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP). Inhibition P2Y11 purino-receptor with NF340, prevented the ASL height increase induced by LXA_4. The ASL height increase and ATP release induced by LXA_4 were both blocked by the ALX/FPR2 receptor inhibitor, Boc-2. Our lab has previously reported the increase in tight junction formation in non-CF bronchial epithelium;

however, the effect of LXA_4 on CF tight junctions had yet to be reported. We examined the effect of LXA_4 on tight junction formation in CF bronchial epithelium and the resulting physiological effects it may have on bacterial invasion and epithelial integrity. Using a gentamicin exclusion assay, confocal microscopy and Western blotting, we investigated the role of LXA_4 on epithelial integrity of bronchial epithelial cells when infected with *Psudomonas aeruginosa (P. aeruginsosa)*. Exposure of *P. aeruginsosa* to bronchial epithelial cells decreased the amount of ZO-1 protein (integral member of tight junction complex) synthesised and induced disruption of tight junctions. LXA_4 not only increased ZO-1 protein synthesis but also prevented the decrease in expression of ZO-1 proteins and tight junction disruption induced by *P. aeruginsosa* in both non-CF and CF bronchial epithelium. Pre-treatment with LXA_4 for 24 h prior to inoculation with *P. aeruginosa*, significantly delayed the invasion of non-CF and CF bronchial epithelial cells by *P. aeruginosa*, while LXA_4 alone did not affect the growth of *P. aeruginosa.*

This thesis provides evidence for a novel effect of LXA_4 in mediating physiological responses that result in the protection of CF bronchial epithelial cells from bacterial invasion.These novel findings may open a new therapeutic avenue for suffers of CF lung disease.

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List of Abbreviations

- ^{10}Panx inhibitory peptide of pannexin-1
- 12-LO 12-lipoxygenase
- 15-LO 15-lipoxygenase
- 15-HETE 15-Hydroxyeicosatetraenoic acid
- A2B adenosine receptor
- ABC ATP-binding cassette
- AC adenylate cyclase
- ADP adenosine diphosphate
- AMP adenosine monophosphate
- AJ adherens junction
- ALI Air/Liquid Interface
- ALX/FPR2 formyl-peptide receptor 2
- APS ammonium persulphate
- ASL airway surface liquid
- ATL aspirin triggered lipoxins
- ATP adenosine triphosphate
- BAL broncho-alveolar lavages
- BCA bicinchoninic acid
- BEGM Bronchial Epithelial Cell Growth Medium
- BSA bovine serum albumin
- Ca^{2+} calcium
- CaCC calcium activated chloride current
- cAMP Cyclic adenosine monophosphate
- CBX carbenoxolone
- CF cystic fibrosis
- CFTR cystic fibrosis transmembrane regulator
- CFU/ml colony forming units per millilitre

• CHO	Chinese hamster ovary
• Cl^-	chloride
• DAG	diglyceride
• DPBS	dulbecco's phosphate-buffered saline
• EMEM	Eagle's minimal essential medium
• EDTA	Ethylenediaminetetraacetic acid
• ENaC	epithelial sodium channel
• ERK	extracellular signal regulated kinases
• FBS	Fetal Bovine Serum
• g	grams
• GK	guanylate kinase
• h	hour
• HBE	human bronchial epithelium
• IP_3	inositol triphosphate
• JAM	junctional adhesion molecule
• LB	Luria broth
• LXA_4	lipoxin A_4
• min	minute
• MSD	membrane spanning domain
• MTT	3,-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltet razolium bromide
• MUC	mucins
• Na^+	sodium
• NaCl	sodium chloride salt
• NBD	nucleotide binding domain
• NOCO	nocodazol
• NS	non-stimulated
• P2X, P2Y	purinergic receptors
• Panx1	pannexin-1
• *P. aeruginosa*	*Pseudomonas aeruginosa*

• PBS	Phosphate buffered saline
• PCL	periciliary liquid layer
• PIP_2	phosphatidylinositol 4,5-bisphosphate
• PKA	protein kinase A
• PKC	protein kinase C
• PLC	phospholipase C
• PMN	polymorphonuclear leukocytes
• PROB	probenecid
• PTC	premature termination codons
• RB2	reactive blue 2
• RvD1	resolvin D1
• SDS	sodium dodecyl sulphate
• SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
• SEM	standard error of the mean
• TA39	lipoxin A_4 stable analogue
• TEER	transepithelial electrical resistance
• TEMED	tetramethylethylenediamine
• TJ	tight junction
• UDP	uridine diphosphate
• UTP	uridine triphosphate
• µl	microlitrer
• µg	micrograms
• w/v	weight per volume
• w/w	weight per weight
• ZO	zonula occluden
• °C	degrees celcius
• Δ	Delta

Publications

Lipoxin A_4 Stimulates Calcium-Activated Chloride Currents and Increases Airway Surface Liquid Height in Normal and Cystic Fibrosis Airway Epithelia.
Valia Verrière, Gerard Higgins equal contributor, Mazen Al-Alawi, Richard W. Costello, Paul McNally, Raphaël Chiron, Brian J. Harvey, Valérie Urbach
PLoS ONE 2012 7(5): e37746. doi:10.1371

Activation of P2RY11 and ATP release by LXA_4 restores the airway surface liquid layer and epithelial repair in cystic fibrosis.
Gerard Higgins, Paul Buchanan, Marianne Perriere, Mazen Al-Alawi, Richard Costello, Valia Verriere, Raphael Chiron, Paul McNally, Brian J Harvey and Valérie Urbach (American Journal of Respiratory Cell and Molecular Biology) in press.

Urbach V, Higgins G, Buchanan PJ, Ringholz F. The role of Lipoxin A4 in Cystic Fibrosis Lung Disease. Volume No: 6, Issue: 7, March 2013, e201303018, http://dx.doi.org/10.5936/csbj.201303018 (review, *in press*)

Ringholz F, Higgins G, Buyck JM and Urbach V.
Modulation of airway epithelial cell calcium and airway hydration in cystic fibrosis: Role of Glucocorticoids and Lipoxins. In New developments in Calcium. Ed Masayoshi Yamaguchi; 2014 Nova Science Publishers, Inc. ISBN: 978-1-62948-601-7 (review, *in press*)

Manuscripts submitted and under revision

Physiological levels of lipoxin A4 inhibit ENaC and restore airway surface liquid height in cystic fibrosis bronchial epithelium.
Mazen Al-Alawi, Paul Buchanan, Valia Verriere, Gerard Higgins, Olive McCabe, Richard Costello, Paul McNally, Valerie Urbach, and Brian Harvey (Journal of Physiology)

Communications

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G. Higgins, P. McNally, BJ. Harvey, V. Urbach.
Lipoxin A_4 Delays the Invasion of Human Bronchial Epithelial and Human Cystic Fibrosis Bronchial Epithelial Cells by the pathogen Pseudomonas aeruginosa

35th ECFS Dublin 6th - 9th June 2012, Dublin, Ireland

G. Higgins, P. McNally, BJ. Harvey, V. Urbach .
Airway Surface Liquid layer height in Cystic Fibrosis Bronchial Epithelial cells is increased by LXA_4 via an apical ATP release activating a P2Y receptor pathway.

5th Irish Epithelial Physiology Group, 25th-26th October 2012, Kilkenny, Ireland

G. Higgins, P. McNally, BJ. Harvey, V. Urbach.
Lipoxin protection of airway epithelium from invasion byPseudomanas aeruginosa in Cystic Fibrosis

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G. Higgins, P. McNally, BJ. Harvey, V. Urbach.
Lipoxin A_4 protects airway epithelium from invasion byPseudomonas aeruginosa in Cystic Fibrosis.

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G. Higgins, P. McNally, BJ. Harvey, V. Urbach.
Lipoxin A_4 Delays the Invasion of Cystic Fibrosis Bronchial Epithelial Cells by the pathogen Pseudomonas aeruginosa.

Abstracts accepted for presentation at national and international conferences

112th ASM General Meeting, 16th-19th June, 2012 San Francisco, USA

G. Higgins, P. McNally, BJ. Harvey, V. Urbach.
Lipoxin A_4 increases airway surface liquid height and delays the Invasion of Human Bronchial Epithelial and Human Cystic Fibrosis Bronchial Epithelial Cells by the pathogen Pseudomonas aeruginosa.

Royal College of Surgeons in Ireland, research Day, April, 2012 Dublin, Ireland.

G. Higgins, P. McNally, BJ. Harvey, V. Urbach.
Lipoxin A_4 increases the Airway Surface Liquid layer height in Cystic Fibrosis Bronchial Epithelial cells via an apical ATP release activating a P2Y receptor pathway.

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Royal Academy of Medicine in Ireland (Section of Biomedical Sciences, 20th of June 2013, University College Cork, Ireland

G. Higgins, P. McNally, BJ. Harvey, V. Urbach.
Lipoxin A_4 Delays the Invasion of Cystic Fibrosis Bronchial Epithelial Cells by the pathogen *Pseudomonas aeruginosa*

Awards

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Lipoxin A_4 increases the Airway Surface Liquid layer height in Cystic Fibrosis Bronchial Epithelial cells via an apical ATP release activating a P2Y receptor pathway.

Chapter I - Introduction

1.1 Thesis Introduction

The airway surface liquid layer (ASL) is a thin aqueous film covering the bronchial epithelium. The maintenance of an optimal ASL height for ciliary beating is crucial for adequate mucociliary clearance. The loss of chloride (Cl^-) transport caused by the mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel gene, results in dehydration, mucoidy and reduction in the height of the ASL which favour chronic lung infection and neutrophil based inflammation leading to lung destruction and early death of the cystic fibrosis (CF) patient (Figure 1.1). It is imperative to understand the molecular mechanisms which maintain a normal ASL height under physiological conditions and to identify and characterise molecules that can increase the ASL height in the CF lung. The endogenous lipid ant-inflammatory/pro-resolution mediator, lipoxin A_4 (LXA_4), is one such molecule. This thesis describes physiological effects of LXA_4 on bronchial epithelial ion transport and dynamic regulation of ASL height, tight junction formation and protection of the epithelial layer from invasion by the pathogen *P. aeruginosa*.

LXA_4 was first described by Charles Serhan and has been reported to show decreased levels in the lungs of CF patients (1-4). The physiological role that LXA_4 plays in the lungs of CF patients was explored in this thesis. In the results section of Chapter III, we show using live cell imaging and confocal microscopy, that LXA_4 increased the ASL height in CF bronchial epithelial cell lines and in primary cultures and this effect was sustained for up to 24 h post stimulation. The molecular mechanisms for LXA_4 effects on ASL were also identified and discussed in Chapter IV. LXA_4 was shown to increase the ASL height by a cellular signaling mechansim involving an apical ATP release, activation of a purinoreceptor pathway, in particular the P2Y11 receptor, which stimulated an increase in cyclic adenosine monophosphate (cAMP) and intercellular calcium (Ca^{2+})

In Chapters V and VI we explored additional effects of LXA_4 in protecting bronchial epithelial cells and the epithelial barrier integrity during infection. It has been previously reported that LXA_4 increases tight junction formation in non-CF bronchial epithelium. Chapter VI illustrates (by immunofluorescence and Western Blotting) the increase in tight junction proteins in CF bronchial epithelium induced by LXA_4. By developing an infection model with fully differentiated bronchial epithelium from CF cell lines, primary CF bronchial epithelium cells and the pathogen *Pseudomonas aeruginosa* we investigated further if the increase in tight junction protein expression could protect the bronchial epithelium during infection. We show that the increase in tight junctional proteins stimulated by LXA_4 was directly correlated with a decrease in *P. aeruginosa* invasion of bronchial epithelial cells. The decrease of invading bacteria may provide far reaching benefits for the CF suffer, as chronically infected lungs by the pathogen *P. aeruginosa,* is a hallmark of progressive lung disease. Therefore this body of work provides evidence for LXA_4 as a novel therapeutic treatment of CF lung disease.

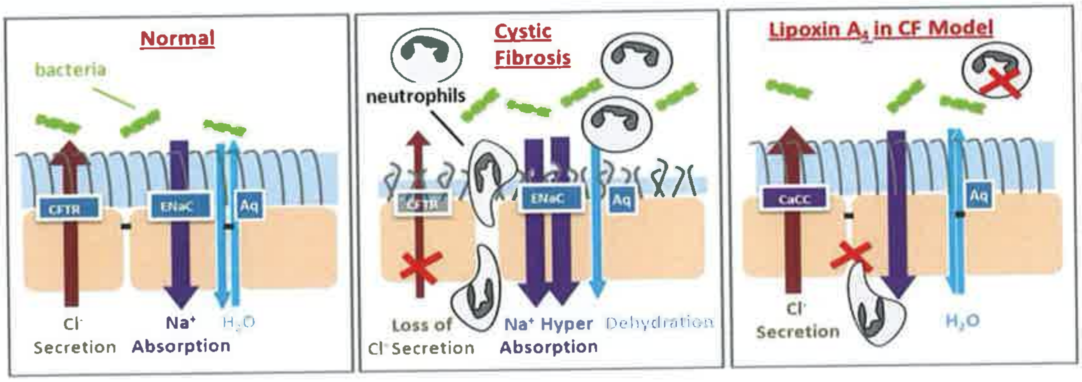


Figure 1.1: LXA_4 increases ASL height and tight junction barrier function in CF bronchial epithelium. In normal airways the ASL provides an adequate mucociliary clearance which is maintained by a combination of Cl^- secretion through the cystic fibrosis transmembrane conductance regulator (CFTR), Na^+ absorption via the epithelial sodium channel (ENaC) and water transport through a paracellular pathway and membrane bound aquaporins (Aq). In CF, a defective CFTR leads to loss of Cl^- secretion and Na^+ hyperabsorption. The concommitant dehydration of the airway lumen favours bacterial infection and inflammation (mainly neutrophilic). This thesis describes how LXA_4 mediates an increase in ASL height and restoring it to normal levels in CF bronchial epithelium. We also describe the effect of LXA_4 to increase tight junction formation, re-establishing the epithelial barrier function and delaying the invasion of the bacteria *P. aeruginosa*. Taken together this work provides evidence for LXA_4 as potentially a new therapy for CF patients.

1.2. General Introduction

1.2.1. Cystic Fibrosis

CF is the most common lethal genetic disorder in Caucasians caused by a mutation in the gene encoding for the CFTR. The disease was first described by Dorothy Hansine Anderson in 1953 who detailed the characteristic cystic fibrosis of the pancreas and correlated it with the lung and intestinal disease that is prominent in CF (5). In 1953, the observation of excessive salt loss in the sweat of CF patients was noted however, it wasn't until 1983 when it was first shown that suffers of CF displayed deficient chloride efflux in sweat but this discovery was not sufficient for the identification of the defective protein in CF patients. In 1985 identification of polymorphic markers in close proximity to the disease mapped the gene to chromosome 7 and then finally to the CFTR (6-8).

The CF gene comprises of 27 coding exons, spanning over 250kb on chromosome on 7q31.2 and the transcript 6.5kb. A sequence comparison between alleles from patients and their parents revealed the major mutation, a 3-bp deletion causing a loss of phenylalanine at position 508 of the protein, designated ΔF508 (9).

The CFTR protein is a chloride channel localised in the apical membrane of exocrine epithelial cells (10). CFTR contains 1,480 amino acids with a molecular weight of ~170kDa comprised of five domains (figure 1.2) , two membrane spanning domains MSD1 and MSD2 that are each composed of 6 transmembrane segments TM1 to TM12 that form the channel, two nucleotide binding domains NBD1 and NBD2 capable of adenosine-triphosphate (ATP) hydrolysis and a regulatory domain (10, 11). The CFTR protein is part of the ATP-binding cassette (ABC) transporter proteins. Consistent with the structure of an ABC transporter protein, it was found that phosphorylation of sites in the regulatory R domain by protein kinase A and the hydrolysis of ATP by NBDs is essential for activation of the CFTR channel (12, 13).

1.2.2. Function of CFTR

CFTR is principally expressed in the apical membrane of epithelia where it provides a pathway for Cl^- and bicarbonate (HCO^-_3) movement and controls the rate of fluid flow through its role as an anion channel while also regulating the function of other ion channels and transporters in epithelial cells (14) . The importance of the CFTR is highlighted by the consequences of CFTR malfunction in CF. Some of the CFTR's distinguishing characteristics are i) it has a small single-channel conductance, ii) the current-voltage relationship of CFTR is linear and iii) the activity is dependent on phosphoryaltion and intracellular nucleotides (11). Another characteristic of the CFTR is that with most ABC transporters the two membrane spanning domains assemble to form a translocation pathway that shuttles substrates across the cell membrane. The MSDs of the CFTR form an anion-selective pore through which anions stream across the cell membrane driven by the transmembrane electrochemical gradient (11).

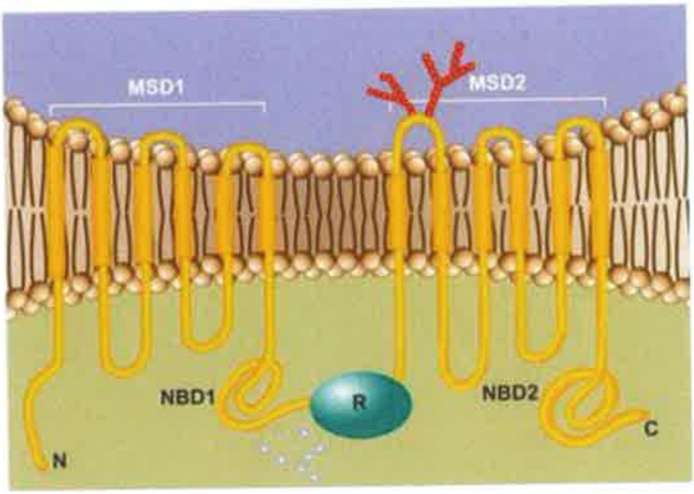


Figure 1.2: Cystic Fibrosis Transmembrane Receptor.

CFTR channels are found in the membrane of epithelial cells throughout the body, where they play a critical role in fluid and electrolyte transport. CFTR is made up of five domains: two membrane spanning domains (MSD1) that form the Cl^-ion channel, two nucleotide binding domains (NBD) that bind and hydrolyse ATP and a regulatory domain (R). Adapted from Lubamba *et al 2012* (15).

1.2.3. CFTR mutations

There are a number of different mutations in the CFTR which lead to differing outcomes in protein synthesis, regulation and CFTR levels within the cell (Figure 1.3). The different mutations and there functional defects are discussed below

1.2.3.1. Class I – Defective Protein Synthesis

Class I mutations are those that include mutations which lead to the disruption of the CFTR protein synthesis. The mutations in this class include non-sense and frame shifts, which lead to the creation of premature termination codons (PTCs). Therefore, such mutations are expected to produce little or no protein. Genotype-phenotype studies of the CFTR revealed that PTCs are associated with a severe form of the disease (16).

1.2.3.2. Class II

With completion of the CFTR protein translation, the abnormal protein undergoes a series of processes in the endoplasmic reticulum and the golgi apparatus. This includes glycosylation and folding that enables protein trafficking to the apical cell membrane. Class II mutations cause impairment of this process, which leads to a degradation of the abnormally processed protein. The major mutation ΔF508, results in the synthesis of a CFTR protein that is unable to correctly fold into its appropriate tertiary confirmation and is retained in the endoplasmic reticulum and degraded (17).

1.2.3.3. Class III and Class IV – Defective Protein Regulation and Altered Conductance

Phosphorylation and dephosphorylation of the CFTR is considered the major pathway by which the Cl^- channel activity is physiologically regulated. Class III mutations include mutations that lead to production of proteins, which reach the plasma membrane; however, their regulation is defective and because of this, they cannot be activated by ATP or cyclic adenosine monophosphate (cAMP). Class IV mutations are associated with an altered conductance with the rate of chloride transport reduced. Mutations in both Class III and Class IV lead to CFTR proteins that can be produced, processed, transported and inserted into the apical membrane but have a defective conductance [13].

1.2.3.4. Class V – Reduced CFTR level

Class V mutations lead to the production of normal proteins, however at reduced levels. This class includes promoter mutations that reduce transcription and amino acid substitutions that cause inefficient protein maturation (18).

1.2.3.5. Class VI – Decreased stability or altered regulation of separate ion channels

In class VI mutations, although the CFTR protein is functional, it is however unstable at the cell surface and is quickly removed and degraded. This mutation leads to a inherent dysfunction of the CFTR protein and its ability to regulate ion movement across epithelial cell membranes.

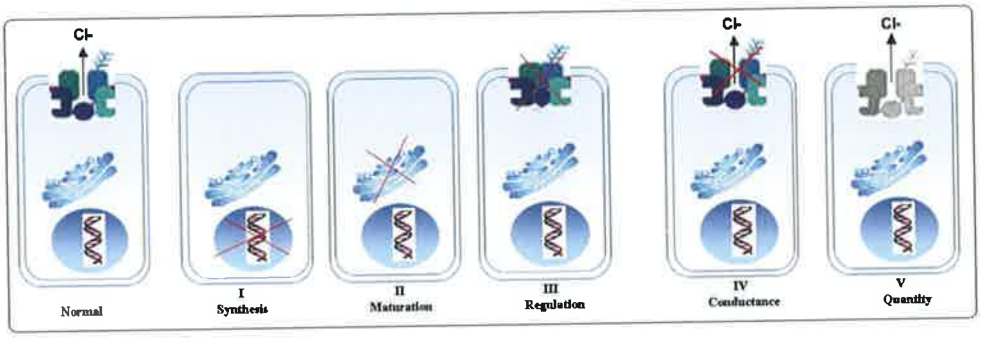


Figure 1.3: Physiological impact of the most common CFTR mutations. The different CFTR mutations can be divided into six major classes according to their effect on the CFTR function. Adapted from Tsui, 1992 and modified by Welsh and Smith (1993) (19, 20)

1.2.4. CF treatments

CF patients are generally treated with medication for the symptoms of CF disease including antibiotics to fight infections, beta-agonists to prevent wheezing and breathlessness (21), inhaled steroids to reduce the lung damage due to inflammation and antibiotics and/or antifungal agents to eradicate infection (22, 23). Further treatments used are aerosolised recombinant DNase (rhDNase) which works by cleaving extracellular DNA that is released from cells during necrosis, decreasing the viscosity of the mucus build up in the CF lung and has proven to improve lung function and reduce exacerbations (24). Another aerosolised therapy using hypertonic saline (3% and 7% sodium chloride (NaCl) has been demonstrated to reduce mucus burden enhancing the clearance of the lung. Hypertonic saline works by causing a dissociation of DNA from the mucus layer but also dilutes the mucus layer by hydrating the airways, restoring the mucociliary clearance removing mucus and foreign bodies from the lung (25, 26). It has previously been noted that use of rhDNase and hypertonic saline as a combination therapy significantly reduces mucus viscosity within the CF lung (27).

There are two drugs available that treat the cause of CF. Firstly, a potentiator drug called Ivacaftor (VX-770) produced by Vertex Pharmaceuticals (MA, USA). VX-770 is used to open the CFTR channel and is currently administered orally in patients with the G551D mutations alleviating the lung function exacerbations associated with gating defects of the CFTR in these patients (28, 29). Secondly, another drug developed by Vertex Pharmaceuicals is the VX-809 which is a corrector type drug enables the trafficking of the CFTR to the cell surface and may have the potential to treat patients with the ΔF508 mutation as bronchial epithelial airway cells isolated from ΔF508 patients treated with VX-809 have an increased presence of CFTR on the plasma membrane (30).

1.2.5. CFTR expression

CFTR shows widespread expression in epithelial cell membranes and the loss of functional CFTR corresponds well with the sites of CF disease such as the submucosal glands and airway surface epithelium (31), pancreatic ductal epithelium, the epithelium of the crypts of Lieberkuhn throughout the gastrointestinal tract (32), the epithelium of sweat glands (33), the epithelium of the developing genital ducts, adult epididymis and vas deferens and the cervical and utrine epithelial surfaces (34, 35). However, there are exceptions to the occurrence of CF disease in epithelial tissues where CFTR is present such as kidney collecting ducts, the epithelium of Burners gland and the submucosal glands of the duodenum (35).

It was widely regarded that pulmonary complaints of CF was a direct consequence of the dysfunctional CFTR in epithelial cells. It is now argued that other cell types such as neutrophils (36, 37), macrophages (38, 39) and dendritic cells (40) are directly affected by the absence or dysfunctional CFTR. The most controversial cell type involved is the neutrophil with conflicting evidence in the literature about the presence of CFTR and the role it may play in neutrophils. Two groups have reported that CFTR is not present in the neutrophil as determined by Western Blotting and Immunofluorescence techniques (38, 41). However there are also a number of reports stating that CFTR is present in neutrophils and plays a crucial role in regulating Cl^- transport within the phagosome and may explain the dysfunctional role that neutrophils play in CF and sustained inflammation observed in the CF lung (36, 37, 42).

1.2.6. Loss of CFTR and CF lung disease

Cl^- is secreted by epithelial cells via the apical CFTR Cl^- channel with Cl^- entering the cell through the Na+-K-2Cl co-transporter localised in the basolateral membrane. Regulation of Cl^- secretion determines the net transport of ions across the epithelium and hence the mass of salt on the epithelial surfaces (22). CFTR was also found to regulate the epithelial Na^+ channel (ENaC) and inhibited its response to phosphorylation by protein kinase A (PKA) suggesting that CFTR acted both as a Cl^- channel and as a regulator of other ion transport processes most notably sodium transport. Indeed, *in vivo* reconstitution of CFTR function in airway epithelia via-adenovirus mediated expression of a normal CFTR gene resulted not only in a restored Cl- channel activity but also inhibited Na+ hyperabsorption (23).

1.2.7. Epithelial Na^+ channel ENaC

The absorptive pathway reflects the capacity to actively absorb Na^+ ions entering the airway epithelial cells from the lumen through ENaC with Na^+ ions exiting the cell via the basolateral Na^+-K-ATPase with Cl^- following passively through the tight junctions (43). ENaC is a hetromultimer that is composed of two α one β and γ subunits that is apically located and amiloride sensitive. In CF airways a defective CFTR leads to an enhanced Na^+ absorption via ENaC. In normal human airways ENaC is inactivated by agonists which raise cAMP such as forskolin (44) and these agonists also activate CFTR induced Cl^- secretion. Microelectrode analysis identified amiloride sensitive apical membrane Na^+ conductance as the rate limiting step for Na^+ absorption and revealed a 2-3 fold increase in apical membrane amiloride sensitive conductance in CF airways (45).

1.3. Airway surface liquid

CF lung disease reflects the failure of airway defence against chronic bacterial infection. Studies in CF airway epithelium cultures, transgenic mice and CF patients suggest that the initiating event in CF airway disease is a reduced ASL volume resulting from dehydration of the airways. This dehydration leads to reduced mucus clearance, adhesion of mucus to airway surfaces and chronic bacterial infection of the lung (44, 46-51).

Mucus clearance is a primary form of pulmonary defence and the efficiency of mucociliary clearance in large part depends on the volume of ASL which makes up the periciliary liquid layer (PCL) and the mucus layer. The mucus layer is an unrestrained tangled gel generated by high molecular weight proteins including secreted MUC5AC and MUC5B. The PCL is closer to the epithelium and its role is to lubricate the cell surface. It provides a low viscosity solution for ciliary beating and mucus transport (49). The lung must continually defend itself against bacteria that deposit on the airway surfaces during normal tidal breathing. The ASL allows for mucus containing foreign bodies and particles to be shuttled along the PCL, up away from the lung to the oesophagus where it is either expelled from the body or swallowed to be digested and destroyed in the gut. However, in the CF lung, the primary pathophysiologic defect is the depletion of the PCL volume due to the lack of functioning CFTR. The decreased PCL volume favours mucus stasis and plugging which leads to persistent bacterial infection, an aggravated immune response, bronchial epithelium remodelling and ultimately lung destruction.

1.3.1 Regulation of ASL by CFTR and ENaC

The hydration of the normal airway surface is maintained in the highly water permeable airway epithelium by active ion-transport processes that controls the quantity of salt (NaCl) delivered to airway surfaces, with water following passively by osmosis (52). The NaCl solution on the airway surfaces is tightly regulated in normal airway epithelia by ENaC mediated Na^+ absorption and Cl^- secretion mediated by CFTR and Ca^{2+} activated Cl^- channels.

Experiments on well differentiated bronchial epithelia grown in air-liquid cell culture models have been key to understanding ASL homestasis. These techniques have given the advantage of studying live airway epithelial cells with an intact native ASL (53). *Tarran et al* (47, 53, 54) showed that non-CF airway epithelia were capable of absorbing an excess of ASL by balancing sodium absorption and chloride secretion to return ASL height to approximately the height of outstretched cilia (7 μM). In CF airway epithelia the Cl^- secretion is lost and Na^+ hyperabsorption is present and airways are unable to regulate ASL height resulting in defective mucociliary clearance as illustrated in figure 1.4. In CF lung disease the absence of CFTR-mediated Cl^- secretion is responsible for the reduced ASL volume. In addition, the inhibitory effect of CFTR on ENaC is lost and Na^+/water absorption proceeds at an unregulated rate predisposing the airway to further loss of ASL (55).

Since the activity of ENaC and CFTR are reciprocally regulated, the demand for increased or decreased ASL volume is met through coordinated changes in ENaC and CFTR activities. The inhibitory effect of CFTR over ENaC in normal airways is central to maintain a relatively constant volume of ASL thereby maintaining mucocillary clearance under a variety of different physiological conditions (26,28). If the ability of normal airway epithelium to secrete Cl^- is inhibited by pre-treating the cultures with an inhibitor of Cl^- secretion e.g. bumetanide, homeostasis of the ASL is lost and the volume of the PCL is decreased. Likewise, if nystatin (antifungal agent, which exhibits pore forming properties as its mode of action) is added to cultures to the apical membrane to bypass the physiological inhibition of ENaC the PCL liquid is also absorbed resulting in CF like absorption rates. Additional evidence that excessive ENaC activation will not only deplete the ASL height but also reduces mucus clearance came from the creation of a mouse model that had an overexpressed ENaC specifically in airway surface epithelia. This resulted in an increase in amiloride-sensitive Na^+ currents which lead to a decrease in ASL height, delayed mucocillary clearance, development of mucus plugs in the airways, airway inflammation and early death of the transgenic mice (56).

1.3.2. Ca^{2+}-activated Cl^- current

An alternative CFTR-independent and Ca^{2+}-activated Cl^- current (CaCC) is also present on the mucosal surface of the airway epithelium and is activated by elevation of cytosolic Ca^{2+} levels. In normal resting conditions, cytosolic Ca^{2+} levels are low and therefore CaCC activity is small. However, strong CaCC activation is associated with conditions promoting Ca^{2+} mobilization from intracellular stores or the influx of extracellular Ca^{2+} (57) . The identity of the CaCC had remained elusive for many years but three independent groups have recently reported that members of the anoddramin ANO/TMEM 16 (TMEM16A) family of membrane proteins are components of the CaCC (57, 58). TMEM16A has biophysical and pharmacological properties that have been attributed to Ca^{2+} dependent Cl^- channels in various tissues with particular importance in epithelial cells. Because of its role for Ca^{2+} dependent Cl^- secretion within epithelial cells the stimulation of CaCC has become a therapeutic target to treat lung and gastrointestinal CF disease.

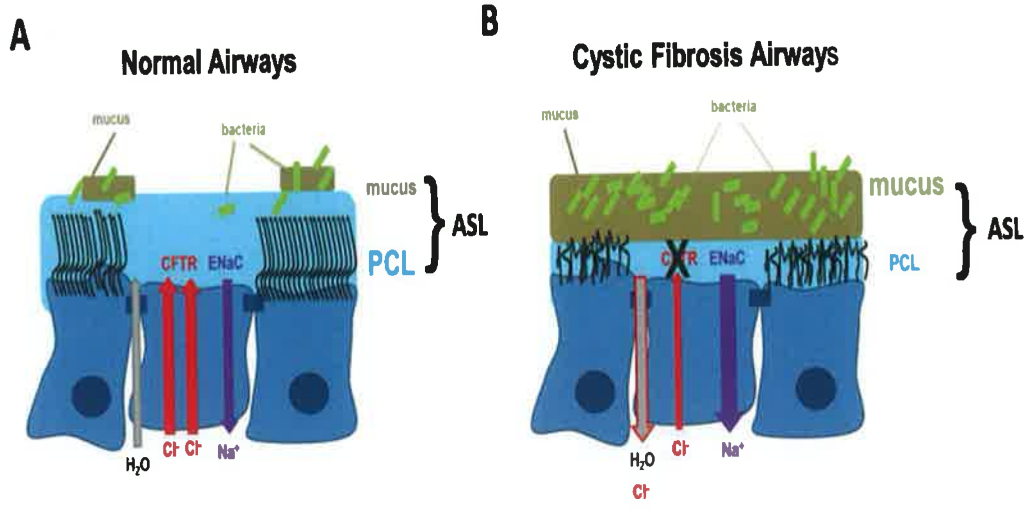


Figure 1.4: Regulation of ASL in normal and CF airways. ASL possesses a mucus component that traps inhaled particles and a liquid layer (PCL) that propels mucus away from the surface of epithelia. (A) The PCL volume is finely balanced in normal epithelial cells by chloride (Cl^-) secretion through the cystic fibrosis transmembrane conductance reulator (CFTR) and sodium (Na^+) absorption through the epithelial sodium channel (ENaC). In CF lungs, the loss of CFTR results in Na^+ hyperabsorption leading to dehydration of the airways. The decrease in PCL liquid layer leads to defective mucociliary clearance, mucus stasis, increased bacterial load and infection resulting in chronic lung destruction (B).

1.3.3. Regulation of ASL by nucleotides and nucleosides

Mason et al first proposed that extracellular ATP regulates ion transport rates when added to either the apical or basolateral surface of human airway epithelium and found that these effects appear to be mediated by cell surface receptors that respond to ATP by regulating ion transport rates through the release of Ca^{2+} from internal stores and extracellular Ca^{2+} influx (59). The airway surface epithelia in the smaller and larger conducting airways are capable of ATP release in response to stimuli such as flow, touch, cyclic nucleotides hypotonicity and Ca^{2+} agonists (60).

As agonists were being screened to restore Cl^- and fluid secretion in CF airway epithelium, nucleotide agonists emerged quickly as stimulants of Cl^- and fluid secretion independent of CFTR. *Knowles et al* showed that extracellular nucleotides stimulated Cl^- secretion in CF patients. Purinerguc agonists in addition to ATP such as UTP, UDP and ADP also had the power to stimulate Cl^- secretion in CF and non-CF airway epithelial models (61). In addition to this work *Tarran et al, and Boucher et al* observed that (62, 63) adenosine receptors could also stimulate Cl^- secretion in airway epithelial cells by activating the cAMP/PKA signal transduction pathway and eventually CFTR. Studies have shown that the concentration of adenosine on airway surfaces under static conditions will activate the A2B purionoreceptors, raise cAMP and activate CFTR sufficiently to provide CFTR-dependent regulation of ENaC and regulation of Cl^- secretion to maintain the optimum 7 μM ASL height. Evidence for the importance of adenosine in the luminal compartment in controlling ASL volume emerged from studies in which an antagonist of adenosine receptors was added apically to airway epithelial cells resulting in the loss in ability to regulate ASL volume (62). In CF the adenosine A2B receptor system and cAMP-dependent activation of PKA are functional, but the absence of CFTR protein in the membrane renders the Cl^- secretory and Na^- inhibitory effects of adenosine signalling ineffective (63).

ATP signalling through purinergic P2Y receptors is effective in airway epithelia in inhibiting ENaC activity and initiating Ca^{2+}-activated Cl^- secretion (64). The concentration of ATP in ASL is determined mainly by the mechanical stresses imparted to airway epithelia during breathing (47). CF airway cultures can lose the ability to transport mucus within 24-48 h but it takes months if not years for mucus plugs to form *in vivo*, which suggests that aspects of CF ASL volume regulation from standard culture systems is missing (65). *Tarran et al* (54) suggested that phasic motion, induced by tidal breathing *in vivo*, was missing from standard static culture systems which regulate ASL height. Exposure of non-CF airway cultures to physiologic levels of shear stress approximately doubles the ASL height, however in CF airway cultures; the ASL height is increased to ~ 7 μM. This effect is mediated in part by increased ATP-dependent activation of P2Y receptors which stimulates Ca^{2+}-activated Cl^- channels to secrete Cl^- and increase ASL height but this effect is approximately half of the increase seen in normal airways which may reflect the lack of a response from CF airways to adenosine on A2B-receptors and CFTR (63).

1.4. Purinergic receptors

Purinergic signalling has now been accepted as a mediator of extracellular communication between cells that include many steps and processes. There are two families of purinergic receptors that are activated by extracellular nucleotides the P2X and P2Y receptors.

1.4.1. P2X receptors

The inotropic P2X receptors (P2X1-P2X7) subunits all have a common toplogy, processing two plasma membrane spanning domains and a large extracellular carboxyl and amino termini (66). Evidence from early molecular and functional studies have strongly indicated that the functional P2X receptor protein is a trimer with 3 peptide subunits arranged around an ion permeable pore. They are involved in a variety of physiological processes; modulation of cardia rhythm and contractility, modulation of vascular tone and contraction of the vas deferens during ejaculation (67). ATP binds to the extracellular loop of the P2X receptor, where it evokes a conformational change in the structure of the ion channel that results in the opening of the ion-permeable pore. This allows cations such as Na^+ and Ca^{2+} to enter the cell membrane and activation of various Ca^{2+} sensitive intracellular processes (68).

1.4.2. P2Y receptors

P2Y receptors are G-protein coupled receptors that stimulate a phospholipase C (PLC)-mediated PIP_2 hydrolysis and activation of the IP_3 pathway. These receptors produce a Ca^{2+} response in which the initial depletion of intracellular Ca^{2+} stores leads to a sustained Ca^{2+} release from the opening of store operated Ca^{2+} channels in the plasma membrane (69, 70). P2Y receptors are G-protein-coupled receptors and have seven transmembrane spanning domains and can be sub-divided into two groups based on their coupling to specific G proteins. Receptors P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 couple to G_q/G_{11} proteins and activate PLC, whereas the P2Y12, P2Y13 and the P2Y14 receptors are coupled to G_i proteins and inhibit adenylate cyclase. The fact that nearly all cells have P2Y-receptors supports the significant physiological relevance of these receptors (71). The P2Y11 receptor has the unique property to couple through both the Gq and Gs proteins and is also the only P2Y receptor of which the coding sequence is interrupted by an intron (72).

A common observation in epithelia is that nucleotides create changes in transpeithelial ion transport rates which can be recorded by the electrophysical technique of short-circuit current I_{sc}. These electrophysical responses are manifested by fast and large transient I_{sc} responses that may not be followed by a secondary slower response. Usually, the I_{sc} response to luminal application of ATP/UTP is stronger than that elicited by basolateral application of the nucleotides. The intracellular Ca^{2+} signals are typically longer lasting and are equally as strong when agonists are added to either epithelial surface (71). The overall I_{sc} response may be the result of multiple P2 receptor stimulation, nucleotide hydrolysis, adenosine receptor stimulation, P2 receptor desensitization as well as time-dependent stimulation of different ion channels and transporters.

Epithelial transport processes are regulated by a large number of hormones, neurotransmitters and local agonists. It is now firmly established that purinergic signalling plays an important role in epithelial transport (71). This was confirmed by *Tarren el al* 2005 which found that ASL height was increased in airway epithelial cells cultured under phasic motion and luminal shear stress. The ASL response was dependent on ATP release and stimulation of the P2Y2 receptor to activate an outward rectifying Cl^- channel (54). Luminally applied UTP/ATP and thus most likely P2Y receptors, increase intracellular Ca^{2+} in many secretory epithelia including respiratory epithelia which may result in purinergic signalling as an important regulatory link that may be exploited therapeutically in CF (71).

1.4.3. P2Y11 receptor

All functionally defined P2Y receptors are able to couple through the phosphatidylinositol 4,5-bisphosphate (IP_3) pathway consisting of activation of phospholipase C (PLC) increase in inositol phosphates and mobilization of Ca^{2+} from intracellular stores. In addition, and secondary to the activation of the PLC, multiple signal transduction pathways including protein kinase C (PKC), phospholipase A_2, Ca^{2+} sensitive ion channels and formation of endothelium derived relaxing factors have been shown to be involved in the responses to activation of native P2Y-receptors.

Pharmalogical data has shown that the P2Y11 receptor is preferentially activated by ATP and is uniquely coupled to both the phosphoinostide and the cAMP pathways (Figure 1.5) (73). Evidence is available that ATP raises cAMP levels in native cells via activation of P2Y11 receptors. This pathway provides a mechanism in addition to activation of P2Y2 or adenosine receptors, by which exogenous or endogenously related nucleotides can increase cellular levels of this important cyclic nucleotide. The nucleotide activation of P2Y11 receptors provides a means for autocrine regulation of epithelial cell function (57). Previous work carried out on the P2Y11 receptor has shown that activation of the receptor by its agonist ATP in different cell types has a number of different outcomes, for example activation of the P2Y11 receptor leads to inhibition of neutrophil apoptosis (73), impairment of cell proliferation in endothelial cells (74) and modulation of pancreatic secretion (75).

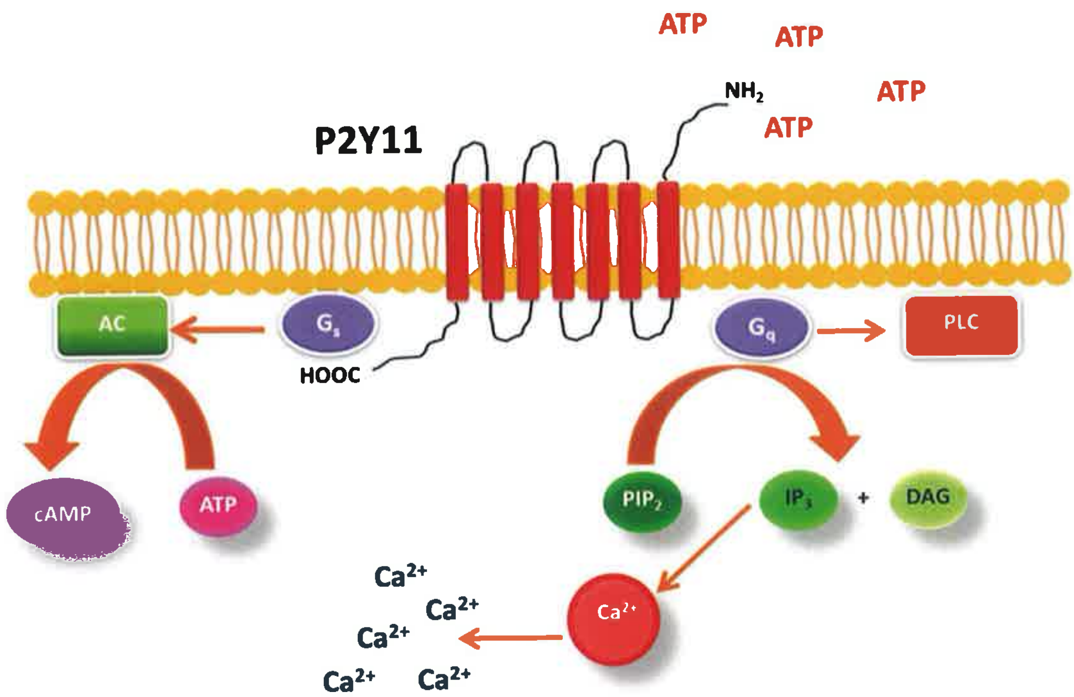


Figure 1.5: Extracellular ATP binds to P2Y11 to activate intracellular Ca^{2+} release and cAMP signalling. Activation of P2Y11 receptors promotes a g-protein (Gq) mediated activation of phospholipase C (PLC) which in turn hydrolysis membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield inositol triphosphate (IP_3) and diglyceride (DAG). IP_3 consequently promotes the release of calcium (Ca^{2+}) from intracellular stores which stimulate chloride (Cl^-) secretion via a Ca^{2+} activated chloride channel. P2Y11 receptors are also coupled to a Gs-mediated activation of adenylate cyclase (AC) increasing concentrations of intracellular cAMP respectively which can inhibit activation of the epithelial Na+ channel (ENaC).

Another function of the P2Y receptors is the activation of ciliary beat frequency. Airway epithelial cells contain hundreds of motile cilia that beat at regular intervals to propel mucus over the epithelial cell surface. In hydrated airways, the rate of mucociliary clearance is determined by ciliary beat frequency (57). Extracellular nucleotides regulate ciliary beating in the airways by mechanisms that involve Ca^{2+} mobilization via P2Y2 receptor activation. The regulation of ciliary beat frequency by P2Y receptors involves a bi-phasic intracellular calcium signaling response. A transient Ca^{2+} signal is associated with Ca^{2+} release from intracellular stores which is followed by a more sustained Ca^{2+} influx via plasma membrane Ca^{2+} channels. Low physiological concentration of ATP generated alternating Ca^{2+} signals in ciliated cells which in turn increases ciliary beat frequency. Maximally effective concentrations of ATP elicited a robust ciliary beat frequency response, which in turn was associated with a sustained Ca^{2+} influx via a receptor operated signal Ca^{2+} entry mechanisms (76).

Some of the above studies highlight the importance of purinergic signalling in the airways. It is clear that adenosine and ATP are crucial regulators of mucociliary clearance in normal airways. P2Y receptors offer promising perspectives as a therapeutic target to promote CaCC activity and ENaC inhibition in the CF airways, improving the otherwise poor ASL volume production associated with defective CFTR.

1.5. Release of nucleotides

The complex cellular composition of the airways i.e. ciliated cells and mucin-secretory goblet cells suggests that several mechanisms and pathways are involved in the release of nucleotides into the airways. Two general mechanisms for the release of ATP from cells have been proposed as vesicular release and channel-mediated release.

Initial evidence for the involvement of the secretory pathway in the release of nucleotides from non-excitatory cells was provided by studies of glucose-dependent ATP release in the yeast *Saccharomyces cerevisia*. A glucose-dependent ATP release was enhanced in yeast overexpressing Mod4p, a gogli-resident transporter predicted to transport ATP to the luman of the secretory pathway. This mechanism of ATP release was inhibited by the Golgi-disrupting agent brefeldin A (57, 77).

Additional evidence for the involvement of the secretory pathway in the release of nucleotides was obtained from the observation that ATP release is accompanied by the release of UDP-sugars (78). A series of studies indicated that UDP-glucose is released by highly differentiated airway epithelium cells. The fact that UDP-sugars participate in glycosylation reactions within the secretory pathway suggested that these molecules are released as cargo molecules during the export of glycoconjugates to the plasma membrane.

Studies on goblet-like airway epithelial cells indicated that ATP and UDP-sugars are released at the same time as MUC5AC, which is the main secretory mucin during Ca^{2+} regulated exocytosis of mucin granules (79). The kinetics of ATP release and mucin-granule secretion are similar and triggered by identical stimuli. This suggests that nucleotides are stored within and released from mucin granules in goblet cells (79). These findings are consistent with the possibility that a vesicular/granular ATP pool contributes to Ca^{2+}-stimulated ATP release. Important observations from this work is that ATP/mucin-secreting goblet cells produce paracrine signals for P2Y2 receptor mediated hydration and mucociliary activities. While vesicular release of ATP is well documented, it cannot be in the exclusive release mechanism, as ATP release can occur in the absence of vesicules. For example, human erythrocytes which are devoid of cytoplasmic vesicles can release ATP in low oxygen content or in response to shear stress as part of a control loop or periheral profusion (80).

1.5.1. Connexin and pannexin channels

Connexin and Pannexin channels have been proposed as diffusion pathways for ATP release under various experimental conditions. Connexin hemichannels are highly dependent on extracellular Ca^{2+}; they close in the presence of milimolar Ca^{2+} and open when the Ca^{2+} is lowered. The pannexins primarily form membrane channels i.e. oligomeric structures embedded in a single plasma membrane, when open provide a conduction pathway between cytosol and extracellular space. Pannexins are better candidates to mediate ATP release than connexins as they are expressed in the right places i.e. in cells that release ATP which is not always the case for connexins, which are mechanosensitive and highly permeable to ATP (81).

1.5.2. Pannexin 1 (Panx1)

Panx1 channels are the most studied of the pannexin family. The gene encoding Panx1 is located on Ilq14.3 and has five exons, encoding at least two alternatively spliced mRNAs. The predicted secondary structure is similar to that of connexions, with four predicted transmembrane domains and intracellular C and N termini (Figure 1.6), leading to the assumption that functional pannexin channels are hexameric (82).

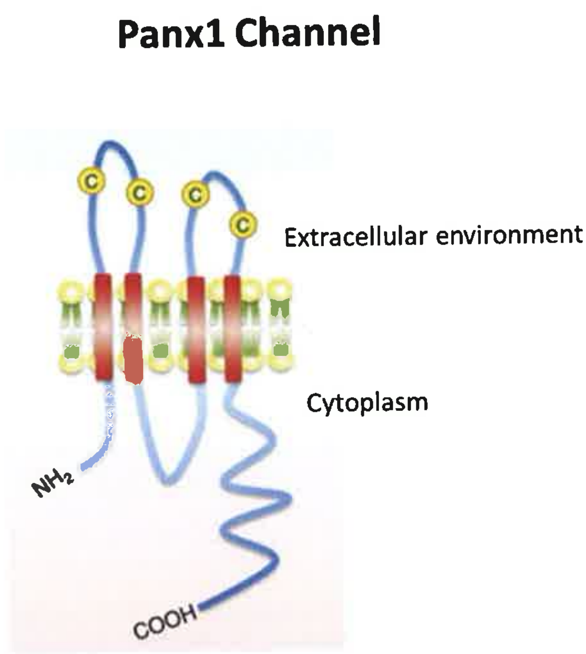


Figure 1.6: Pannexin-1 predicted structure. Pannexin family proteins consist of four transmembrane domains, two extracellular loops and amino- (NH_2) and carboxyl (COOH) terminal cytoplasmic tails with each extracellular loop comprising of three two cysteine residues (C). Adapted from Iwamoto *et al 2013* (83).

The large pores of Panx1 are permeable to ions, second messengers and neurtransmitters such as ATP, IP_3 and amino acids. Panx1 is also implicated in secretion of arachidonic acid (82) and its metabolites and it is now widely regarded that Panx1 membrane channels are also involved in the extracellular mode of wave propagation. Panx1 channels open in response to mechanical stress or other stimuli such as depolarization and release ATP to the extracellular medium . ATP binding to purinergic receptors triggers an increase of cytoplasmic Ca^{2+} via the IP_3 pathway. The Ca^{2+} increase is not restricted to the same cell but also includes cells within the diffusion distance for the released ATP. The autocrine release of ATP also stimulates cells that are coupled to adjacent stimulated cells by gap junction channels permitting the flux of IP_3. The increase in Ca^{2+} can activate Panx1 channels and the subsequent release of ATP provides a new source for extracellular ATP to reach more distant cells (81). The application of micro-molar concentrations of Ca^{2+} to the cytoplasmic side of Panx1 channels in excised membrane patches activated the channels at negative membrane potentials where the channels are normally closed (81, 84).

1.6. Tight junctions

Fluid absorption is controlled mainly by the transport of Na^+ through the apical ENaC channel as described above, while fluid secretion is regulated by the cell by movement of Cl^- through channels such as CFTR or CaCC. The movement of counter ions is likely to take place through tight junctions (TJ) (85). TJs create a rate-limiting barrier to diffusion of solutes between vertebrate epithelial cells and endothelial cells. Located at the apical region of the lateral cell membrane surface, they act as a fence within the membrane bi-layer to separate distinct lipid and protein components of the apical and basolateral domains. Their main function is to act as a barrier to prevent paracelluar diffusion between cells which enables the epithelial monolayer to restrict permeability to solutes or larger particles including pathogens [73]. The barrier can vary in tightness by several orders of magnitude in electrical resistance between tight and leaky epithelia, they show a slight cation selectivity and depending on the cell type have a pore size in the range of 8-20 Ω (86).

To date there have been 40 proteins identified in TJ formation containing a complex protein composition. The TJ structural complexity is a result of its many interrelated roles in cell polarity, signalling, transcriptional regulation, cell cycle, vesicle trafficking and creating a paracellular barrier. TJs contain three classes of essential membrane proteins – occludins, claudins and junctional adhesion molecules (JAMs). The occulidin and claudins form the backbone of tight junctions while JAM is important for the trafficking of white blood cells from vascular compartments to tissues during inflammation. The TJ is attached to the cytoskeleton by a set of adapter proteins one of which is the zonula occuldens protein 1 (ZO-1) (87).

1.6.1. Zonula occuldens

The zonula occludens (ZO) are a family of cyctosolic proteins of three different subtypes ZO-1, ZO-2 and ZO-3 that are multi domain scaffolds that bind and interact with other signalling and structural proteins implicated in TJ structure and function.

1.6.2. ZO-1

ZO-1 is a 210 KDa protein found at the submembranous domain of TJs in epithelia and endothelia. Cells that do not form TJs such as fibroblasts show ZO-1 dispersed in the cytoplasm (88). At TJs ZO-1 is associated through its first PDZ domain to the carboxyl terminal end of claudins, by the second and third PDZs to JAM and by its guanylate kinase (GUK) module to occludin. ZO-2 and ZO-3 independently associate to ZO-1 through a PDZ-2/PDZ-2 interaction. ZO-1 binds to the actin cytoskeleton and to actin binding protein through its carboxyl terminal end (87).

Epithelial barriers create the distinct tissue spaces required for proper organ function. The formation and maintenance of these barriers is dependent on a series of cell-cell contacts that circumscribe the apical-lateral margin of each cell, known collectively as the apical junction complex. This complex includes the adherens junctions (AJ) which promote tissue integrity by establishing a strong adhesive interface between individual cells. Both AJs and TJs are intimately associated with the cortical actin cytoskeleton and functionally regulated by circumferential actomyosin fialments. The dynamic interaction between cell junctions and the cytoskeleton is critical for the morphogenesis of epithelial tissues during development of tissue repair and maintenance in adult organisms (87). *Fanning et al 2012* study revealed that ZO proteins in fully polarized cells regulate the assembly and contractility of the perijunctional actomyosin ring associated with the AJ. The depletion of ZO-1 proteins is associated with an increased contractility of the perijunctional actomyosin ring, increased cell height, dramatic expansion of the apical plasma membrane, and altered cell packing within the monolayer. These observations suggest that ZO-1 proteins play a role in the morphogenetic processes that regulate epithelial organ development and repair (89).

A fully functional epithelium is one of the most fundamental components of the innate immune system, protecting the body from the vast array of microbes. The mucosal epithelium is comprised of polarised epithelial cells with the distinct apical and basolateral surfaces that are defined by a unique set of lipid and protein compositions and separated by a functioning tight junction system. Bacteria and viruses have evolved to circumvent the epithelial barrier by entering into cells or travelling through them by trancytosis, crossing through intracellular junctions or directly disrupting the tight junctions (90).

1.7. Pathogens of the CF lung

The morbidity and death of CF patients are primarily caused by the chronic respiratory infections, resulting in damage and remodelling of the airways which finally leads to pulmonary failure. The chronically colonized lung represents a complex and diverse ecosystem. It is generally regarded that CF patients are colonized by pathogenic microorganisms in infancy and chronic infections are established leading to recurrent acute respiratory failure (91). The prevalence of different bacteria changes over time in the CF lung which is demonstrated in Figure

1.7. The most abundant studied organisms that affect the CF lung are *Haemophilus Influenzae*, *Staphylococcus aures*, *P. aeruginosa* and *Burkholderia*. During the chronic infection of the CF lung, pathogens are subjected to changing selection pressures and they will encounter new habitats and different co-inhabiting/infection species to live along side. The most common observed phenotypic changes of bacteria from the CF lung include antibiotic resistance, an increase or decrease in cell motility, the emergence of mucoid strains, the appearance of small colony variants, increased mutation rate and decreased production or virulence factors associated with acute infections (92). It is reported that the use of antibiotic therapies to eliminate bacterial infection in the lung unintentionally allows fungi to multiply and thrive (97). *Aspergillus* and *Candida spp.* are frequently isolated in CF (98). The clinical significance of *Aspergillus* colonisation has been widely reported, including its role in invasive aspergillosis (99), down-regulation of the vitamin D receptor in macrophages and epithelial cells (100) and allergic bronchopulmonary aspergillosis (100).

Candida is the second most commonly isolated fungal species after *Aspergillus* in the sputum of adults with CF (98); however the clinical consequence is still not fully understood although in 2010, Chotirmall *et al* reported that that *C. albicans* colonisation pre-indicated a decline in lung function and an increase in hospital-treated pulmonary exacerbations (101).

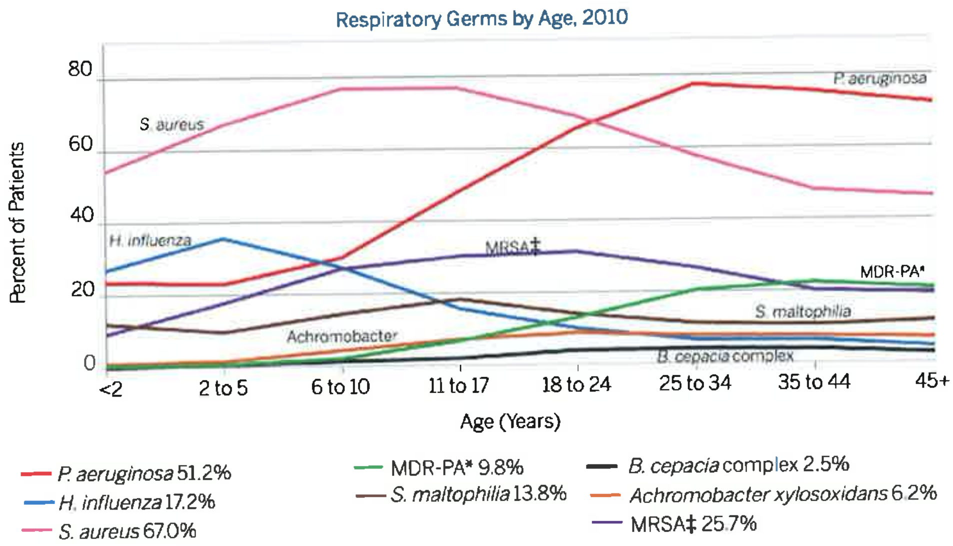


Figure 1.7: Incidence of bacterial infection in the lungs of CF patients. The occurrence of pathogens retrieved from the CF lung. Pulmonary exacerbations in CF are caused by recognised typical pathogens that are acquired following a characteristic age-dependent pattern. Whilst still very young, CF patients suffer their first infections with the bacterium *Staphylococcus aureus* which is often the first organism isolated from children with CF. Non-typeable *Haemophilus influenzae* is commonly cultured from CF children as early as in their first year of life (93-95). Other pathogens in CF usually cultured later in the course of lung disease include *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* with *Burkholderia cepecia* being a signal for increased lung destruction and mortality (96). But the most studied organism that colonise the CF lung is the pathogen *Pseudomonas aeruginosa*. Data from Cystic Fibrosis Foundation Patient Registry, 2010 Annual Data Report.

1.7.1. *Pseudomanas aeruginosa*

P. aeruginosa is an opportunistic pathogen that can be found in soil, water, and most man made environments, using a wide range of organic material to survive. It is a gram negative aerobic bacillus bacterium with unipolar motility. Although classified as an aerobic organism it can and is considered by many as a facultative anaerobe as it is well adapted to proliferate in conditions of partial or total oxygen depletion. *P. aerugionsa* can achieve anaerobic growth with nitrate as a terminal electron acceptor and can also ferment arginine by substrate level phosphorylation. Adaption for microaerobic or anaerobic environments is essential for the growth of *P. aerugionsa*. One example of this requirement is during lung infection of CF patients where thick layers of mucus can limit the diffusion of oxygen (46, 97). The versatility of *P. aeruginosa* enables the organism to infect damaged tissues or those with reduced immunity. If colonization occurs in critical body organs such as the lungs, the kidneys or urinary tract, the results of infection can be fatal.

P. aeruginosa is the most clinically important pathogen in the CF lung. By the time patients reach adulthood, up to 80% become infected with *P. aeruginosa*. The transformation of *P. aeruginosa* from the non-mucoid to the mucoid strains, which often occurs during adolescence years, is pivotal and is known to be associated with clinical and radiological deterioration (98). At initial colonisation, *P. aeruginosa* presents a phenotype similar to environmental strains, but these change dramatically with time and prolonged infection as *P. aeruginosa* has the potent ability of quorum sensing which leads to biofilm formation (99). The formation of alginate biofilm has 3 important consequences. Firstly, the bacteria are protected from antibiotics, which increase the minimal-inhibitory concentration. Secondly, the film reduces the activity of aminoglycosides and beta-lactam antibiotics by changing the pH of the respiratory mucosa and by production of beta-lactamase and thirdly, biofilm formation is highly immunogenic and tends to accelerate structural lung damage leading to significant histological and radiological changes. The emergence of these bacteria is associated with decline in respiratory function (FEV1) and worsening of radiograph appearance (92).

What makes *P. aeruginosa* difficult to deal with in the CF lung is its metabolic versatility, biofilm formation, production of multiple virulence factors and its intrinsic or acquired antibiotic resistance. The virulence machinery of *P. aeruginosa* consists of lipopolysaccharides, pili, and flagella, numerous secreted factors such as elastase, proteases, exotoxins, pyocyonin and extracellular polysaccharides. Acute virulence factors include Type 2 and Type 3 secretion systems, flagella, type IV pili and the use of elastase. Acute infection in the CF lung eventually leads to chronic infection by *P. aeruginosa*, biofilm formation, increase in small colony variants and up-regulation of the Type 6 secretion system. The transition to a chronic infection phase is the result of numerous changes in cellular physiology in response to extreme stimuli. Changes include down regulation of acute virulence genes with an up-regulation of chronic infection phenotypes and antibiotic resistance, host invasion and overproduction of extracellualer polysaccharides (100).

1.7.2. Lung inflammation and resolution

The initial response to inflammation within the lung is characterised by leukocyte infiltration to an inflammatory response under the influence of chemoattractents, frequently generated by pro-inflammatory lipid mediators including leukotriene and prostaglandins. The pro-resolution phase was once thought to be a passive process but it is now generally understood that the resolution of inflammation depends on multiple processes that halt the inflammation process and return tissue and function to its prior state. Among these signals is the lipoxins (LXs) and there aspirin-triggered carbon-15 epimers that were first described by Charles Serhan in 1984 and are key players in the endogenous lipid mediators involved in the resolution of inflammation (101).

1.8. Lipoxin A_4

Lipoxin A_4 (LXA_4) belongs to a class of a newly identified lipid mediators and play a central role in the resolution of inflammation and are often referred to as specialised pro-resolution mediators.

1.8.1. Lipoxin A_4 synthesis

There are two main pathways for LXA_4 synthesis. The first pathway involves lipoxygenation of arachidonic acid by 15-LO in monocytes and epithelial cells and by 5-LO in neutrophils resulting in a reduction of leukotriene formation (102). The second pathway involves the interaction of platelets and leukocytes to induce LXA_4

formation by the activity of 12-LO in platelets and 5-LO in leukocytes (103). Another route for LXA_4 formation involves the esterification of 15-hydroxyeicosatetraenoic (15-HETE) in inositol-containing phospholipids within the membranes of neutrophils suggesting that LXA_4 precursors may be stored within the membranes of inflammatory cells and released after stimulation (Figure 1.8) (104).

Defective LXA_4 biosynthesis has been demonstrated in many human chronic inflammatory diseases including asthma and rheumatoid arthritis. LXA_4 and its positional isomer LXB_4 are the principal species formed in mammals. In a cytokine-primed milieu, aspirin-induced acetylation of cyclooxygenase 2 (COX-2) inhibits endothelial cell prostanoid production and promotes the formation of 15-R-HETE from arachidonic acid to either 15-epi-LXA_4 or 15-epi-LXB_4. These aspirin triggered lipoxins (ATLs) are endogenous 15-R-enantomers of LXA_4 and LXB_4 (105, 106).

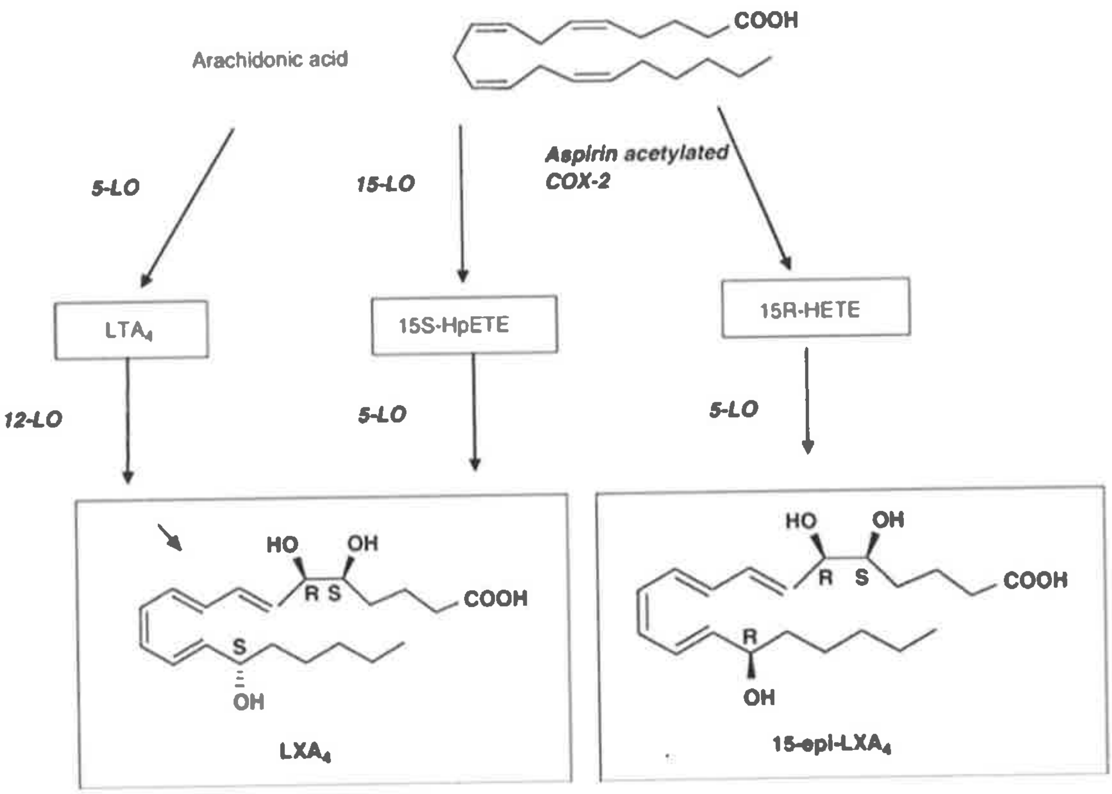


Figure 1.8: Biosynthesis of LXA_4 Arachidonic Acid can be converted into 15(S)-hydroperoxyeicosatetraenoic (15S-Hp-ETE) through 15-lipoxygenase (15-LO) and into 15(R)-hydroperoxyeicosatetraenoic (15R-HETE) by aspirin acetylated COX-2. Both intermediates can be further metabolized through 5-lipoxygenase (5-LO) and enzymatic hydrolysis yielding lipoxin A_4 (LXA_4) or 15-epimer of LXA_4 (15-epi-LXA_4) or leukotriene A_4 (LTA_4) in the presence of 5-LO and 12-lipoxygnase (12-LO) is converted into LXA_4. Adapted from Kowal-Bielecka *et al 2007 (107)*

1.8.1. ALX/FPR2 receptor

LXA_4 binds to the formyl-peptide receptor 2 (ALX/FPR2) with high affinity (108). The formyl-peptide receptors 1, 2 and 3 (FPR1, FPR2 and FPR3) form a subgroup of receptors linked to inhibitory G-proteins (109). Their activation by specific agonists results in transient Ca^{2+} flux, extra cellular signal regulated kinases (ERK) phosphorylation and chemotaxis (110). The seventh transmembrane domain and adjacent regions of the FPR2 receptor are essential for LXA_4 recognition, whereas the additional regions of the FPR2 (e.g. extracellular loops) are required for high affinity binding of the peptide ligands (107). LXA_4 also interacts directly with the cysLTI receptor that also induces signals that prevent the pro-inflammatory responses which contribute to regulating the resolution of inflammation (102, 110)

1.8.2. Actions of lipoxin A_4

LXA_4 has distinct actions on specific leukocytes inhibiting the activation of polymorphonuclear (PMNs) and eosinphils while activating monocytes and macrophages. PMN recruitment is a multistep process that includes chemotaxsis adhesion and transmigration (110). In experimental in-vitro models LXA_4, LXB_4 and ATLS inhibit PMN chemotaxis in response to LTB_4 and inhibit eosinophil responses to platelet activating factor. Stimulation of macrophages with LXA_4 significantly enhances phagocytosis of apoptotic PMN by human monocytes suggesting that LXA_4 can promote the clearance of apoptotic leukocytes by macrophages at an inflammation site (105, 111).

1.8.3. Lipoxin A_4 in CF

The airway of CF is characterised by chronic bacterial colonization and persistent neutrophilic inflammation. In addition to the sustained presence of activated neutrophils and neutrophil derived secretory products, there is also a significant upregualtion of pro-inflammatory cytokine production, IL-8 production, THF and IL-1β are all increased in broncho-alveolar lavages (BAL) fluid, sputa and bronchial biopsies while the production of the anti-inflammatory cytokine IL-10 is suppressed (112-115). Quantification of bacteria and bacterial products also revealed that inflammatory mediators when normalised to the number of bacteria in the airway are significantly increased in children with CF compared to children with other respiratory diseases (116, 117).

The deregulated pro-inflammatory environment of the CF airway is consistent with a deficiency in LXA_4 mediated anti-inflammatory activity. Examination of BAL fluid showed a significant depression of LXA_4/neutrophil ratio in patients with CF compared to those with an inflammatory control group (118). The effect of LXA_4 analogues on infection driven neutrophil accumulation in the airway has also been examined *in vivo*, employing a well characterised mouse model of chronic airway inflammation/infection similar to that seen in patients with CF. The study showed that mice infected with *P. aeruginosa* and treated with LXA_4 when compared to controls had significantly less weight loss along with earlier and more complete recovery of lost weight, an inhibition of airway and lung parenchymal neutrophilia, an indication of a shift from acute to chronic inflammatory condition and a decrease in bacterial burden. The work carried out by Karp *et al* suggests that there is an important pathophysiological defect in LXA_4 mediated anti-inflammatory activity in the CF lung (3). It appears likely that a defect in generation and action of LXA_4 are defected in the CF lung which warrants further examination of LXA_4 biosynthesis and its possible correction as a new therapeutic avenue for CF suffers.

1.9. Aims

The endogenous lipid mediator LXA_4 has been reported to be deficient in the lungs of CF patients. LXA_4 has the ability to down regulate the inflammatory response in normal or non-CF individuals. This decrease in LXA_4 within the lungs of CF patients may have detrimental effects on CF bronchial epithelium and may play a role in the severe inflammation/infection cycle observed. The hypothesis of this study was to determine if the decrease of LXA_4, which is observed in the CF lung, could lead to a physiological disadvantage for the CF patient. Therefore, the major aim of this thesis was to investigate the physiological effects of LXA_4 on CF bronchial epithelium and the molecular mechanisms of lipoxin actions in bronchial epithelium (Figure 1.9).

To fulfil the aim of this study there are four specific objectives, which are as follows:

1. To investigate whether LXA_4 could increase ASL height in CF bronchial epithelial cells

2. To understand the mechanism behind the increase in ASL induced by LXA_4

3. To determine if LXA_4 could prevent the invasion of the airway epithelium by *P. aeruginosa*

4. To determine if LXA_4 can increase the tight junction barrier function to protect bronchial epithelium from invasion by *P. aeruginosa*

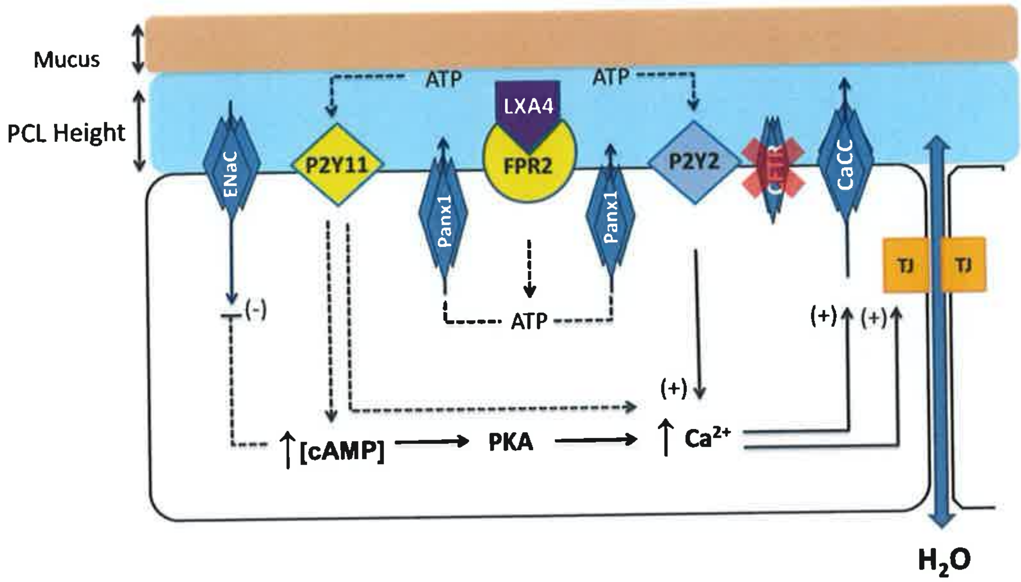


Figure 1.9: Schematic Diagram of hypothesis proposed in this thesis.

The aim of this project is to evaluate the physiological effects of LXA_4 on CF bronchial epithelium. It is hypothesised that activation of the ALX/FPR2 receptor by lipoxin A_4 (LXA_4) induces an apical ATP release through pannexin (Panx1) channel triggering a purinoreceptor pathway increasing both Cyclic adenosine monophosphate (cAMP) and intracellular calcium (Ca^{2+}). The increase in cAMP and Ca^{2+} restore the ASL height by inhibition of sodium (Na^+) absorption through the epithelial sodium channel (ENaC) and stimulation of Cl^- transport through a calcium activated chloride channel (CaCC). It has previously been reported that LXA_4 increases tight junction (TJ) formation non-CF bronchial epithelium cells. It is now proposed that LXA_4 can increase TJ expression in CF bronchial epithelium leading to superior epithelial barrier integrity and delaying the invasion of CF bronchial epithelium by *P. aeruginosa*.

Chapter II - Materials and Methods

In this chapter are presented the materials and methods of the commonly used protocols of each chapter; however, more specific methodologies and protocols related to each individual result chapter may be found within their own chapter.

2.1. Cell lines

NuLi-1 and CuFi-1 cells were donated from Prof. Zabner at the University of Iwoa. The NuLi-1 cell line was derived from airway epithelium of normal genotype. The CuFi-1 cell line was derived from a patient with Δ508/ Δ508 homozygous mutation. CuFi-3 cell line was derived from a patient with the Δ508/R553X. Cell lines were transformed with a reverse transcriptase component of telomerase and human papillomavirus type 16 E6 and E7 genes (119). The development of the NuLi-1, CuFi-1 and CuFi-3 cell lines used in this work has given us the ability to work on a cell model that retained the phenotypic qualities of airway epithelia. This includes the ability to form a mucus secreting electrically tight epithelium. Differentiation of these cells is facilitated by switching from a liquid –liquid interface to air-liquid interface on porous supports which is discussed below in section 2.2 in materials and methods

2.2. Cell Culture

2.2.1. Bronchial epithelial cell growth medium

To 500ml of Bronchial Epithelial Cell Basal Medium (BEGM) (Lonza Clonetics® 500mls CC-3171), BEGM® Single Quots Kit® (Lonza Clonetics® CC-4175 which contain Bovine Pituitary Extract 2 ml, Insulin 0.5 ml, Hydrocortisone 0.5 ml, Gentamicin / Amphotericin -1000 0.5 ml, Retinoic Acid 0.5ml, Transferrin 0.5 ml, Triiodothyronine 0.5 ml, Epinephrine 0.5 ml and Human Epidermal Growth Factor 0.5 ml were added (discarding the gentamicin-amphotericin B aliquot)). 500 µl Gentamicin 10 mg/ml (Gibco®) and 2.5 ml fungizone (Gibco®) were added to the BEGM media. The solution was mixed by inverting and placed in 50 ml tubes at 4^0C until ready for use.

2.2.2. Day 2 media

DMEM - Dulbecco's Modified Eagle Medium 500mls – (Gibco®) and F12 Nutrient Mixture (HAM) 500 ml – (Gibco®) were mixed together. To this mixture 10 ml Penicillin - Streptomycin 100X Solution (Gibco®) (5000 units of penicillin and 5000 µg of streptomycin per ml), 5 ml Fungizone® Antimycotic liquid 250 µg /ml amphotericin B (Gibco®), 1 ml Gentamicin, 10 mg/ml (Gibco®) and Ultroser™ G serum substitute – suspended in 20 ml Sterile Water (PALL Life Sciences) were added. The combined mixture was filter sterilized aliquoted to 50 ml tubes and placed at 4^0C until ready for use.

2.2.3. Collagen coating

50 mg of Collagen from human placenta, Bornstein & Traub Type IV was dissolved (Sigma-Aldrich®, USA) in 83.3 ml of sterile distilled water with 166.6 µl of glacial acetic acid. Collagen stock was diluted 1:10 with distilled water and filter sterilised before use. This is a working concentration of 60 µg/ml of collagen which was used for coating membrane surfaces. The surface of the plastic culture dishes was coated for a minimum of 18 hours at 37^{0}C. The collagen solution can remain on the plastic surface for several days and stay stable until required. Liquid collagen was removed from the surface of the plastic and allowed to air-dry. Plastic surface of the culture dishes were washed at least twice with sterile phosphate buffered saline (PBS) to remove all traces of the collagen. Residual liquid collagen is toxic to cells. Different amounts of collagen was required for different cell culture growth conditions

5 ml for T75 (Corning® Flask)

0.5 ml per well for 6 well Costar® Plates (3513)

0.2 ml per filter for Hanging Filters (Millicell Hanging Cell Culture Insert, Millipore).

2.2.4. Seeding cells from frozen vials

Flasks were collagen coated in advance for minimum of 18 h and rinsed twice in PBS before use. BEGM media was warmed at 37^{0}C in a waterbath (Grant SubAqua 18) for about 15 min. The desired vial of cells was removed from liquid nitrogen and allowed to thaw to 37^{0}C in the waterbath for 1 min or until defrosted.

Once the cells had thawed they were added to 5 ml of BEGM media and centrifuged at 260 g for 5 min at 4°C. The supernatant was removed and the pellet was resuspened in 1 ml of BEGM media. The cell suspension was added to the final desired amount of media and labelled. Cells were placed at 37 °C in 5% CO_2 (Sanyo CO_2 Incubator) feeding every other day with 9-12ml of BEGM media until ~80% confluency was reached which was assessed by microscopy (Olympus CKX 41).

2.2.5. Splitting cells

Cells were washed with 10 ml sterilised dulbecco's phosphate-buffered saline (Gibco®) at least twice and Incubated with ~5-7 ml of Trypsin/EDTA (Gibco®) at 37°C (Sanyo CO_2 Incubator) for 5 min. Once detached, 1ml Fetal Bovine Serum (FBS) (Gibco®) was added to the flask as a trypsin neutralizing solution (FBS 1/10 dilution). Detached cells were transferred from the flask to single a 15 ml tube and cells were pelleted (Eppendorf centifuge 5810R) at 260 g at 4°C for 5 min. Cells were resuspended in 5 ml BEGM media and cell suspension was apportioned for use in transwells or 6 well plates as required. The remainder of the cells was returned to a new T75 flask making a total volume of 10-12 ml.

2.2.6. Seeding cells onto inserts

The collagen coated transwells were washed with PBS at least twice to remove any traces of residual collagen. 250 µl of cell suspension was obtained as described in section 2.2.5. of materials and methods were placed into the hanging transwell. 1.5 ml of BEGM, warmed to 37^0C in the waterbath (Grant SubAqua 18), was pipetted into each well in contact with the basolateral side of the membrane. 250 µl of BEGM was added apically to the cell suspension totalling the apical volume to 500 µl and cells were then incubated at 37^0C in 5.0% CO_2 (Sanyo CO_2 Incubator). 2-3 days after the seeding of cells into inserts confluence was reached. The basolateral and apical media was discarded and changed to "Day 2 Media". From then on epithelia were fed only with Day 2 media basolaterally and apically. This liquid/liquid interface phase is essential to achieve differentiation. Transepithelial electrical resistance measurements (section 2.2.7. of materials and methods) were recorded twice a week and once cells had reached > 200 Ω/cm2 in liquid/liquid interface were then switched to air/liquid interface (ALI) mimicking that of native bronchial epithelial cells. Cells that had reached >700 Ω/cm2 were considered to be fully differentiated.

2.2.7. Measuring transepithelial electrical resistance (TEER)

Once cells had reached confluence on liquid/liquid interface regular TEER readings are obtained using an EVOMX™ (World Precision Instruments, USA). For Liquid/Liquid interface the probe of the EVOMX™ was placed such that it contacts the media on either side of the membrane and a resistance measurement was taken. Following the TEER measurement the medium was aspirated, discarded and the cells were fed with fresh Day2 media to the apical and basolateral side of the membrane. When measuring TEER on ALI cultures, 0.5 ml of media was transferred from the basolateral compartment into the hanging cell culture insert. TEER was measured as above. Following TEER measurement the medium on the apical side was aspirated, discarded and the cells were fed with 1.5 ml of Day 2 media which was added to the basolateral compartment.

2.2.8. Air liquid interface

The apical media was removed and the basolateral media was replaced with new Day 2 media and cells were incubated at $37^{0}C$ in 5% CO_2. TEER measurements were recorded twice weekly until a sufficient TEER value was reached. A TEER reading above 700 Ω/cm2 was deemed acceptable for fully differentiated well polarised bronchial epithelium. Epithelia were fed only on the basolateral compartment. Day 2 media was only used for ALI culturing, BEGM media does not allow for good differentiating of bronchial epithelium. Day 2 media was added basolaterally every other day to the differing culture plastics; 1.5 ml for 12 mm inserts, 2.0 ml for snapwells and 1.0 ml for small inserts.

2.2.9. Feeding cells

Media was warmed in the waterbath at 37^{0}C. Old media was aspirated and discarded. Cells in flasks were fed 9-12 ml of BEGM media, 6 well plates were fed with 2 ml of BEGM per well and returned to 37^{0}C in 5% CO_2 (Sanyo CO_2 Incubator). Cells on inserts were fed with 1.5 ml of media basolaterally and if required 0.5 ml apically with the pre-determined media which is dependent on cell differentiation.

2.3. Primary tissue culture (from bronchial brushings)

2.3.1. Collection and transport

Bronchial brushings were stored on ice in Eagles Minimum Essential Media (EMEM) (Gibco®) completed with 5 ml Penicillin/Streptomycin (Gibco®), 80 µg tobramycin (Gibco®), 2.5 µg Fungizone (Gibco®) and 5 µg gentamicin (Gibco®). The transport of the sample from the hospital to the laboratory was carried out in the following order: Tubes containing the samples were placed in a secondary container (biohazard bag). The biohazard bag was then placed inside a secondary biohazard bag containing ice and placed inside a carrier container clearly labelled biohazard and closed. The container was not opened until the sample had reached the cell culture suite.

2.3.2. Primary CF bronchial epithelial cell reagent preparation

Primary bronchial epithelial cell growth medium (Primary BEGM) contains Bronchial Epithelial Cell Basal Medium (Lonza Clonetics® 500 mls CC-3171), BEGM® Single Quots Kit® (Lonza Clonetics® CC-4175 which contains Bovine Pituitary Extract 2 ml, Insulin 0.5 ml, Hydrocortisone 0.5 ml, Gentamicin / Amphotericin -1000, 0.5 ml, Retinoic Acid 0.5 ml, Transferrin 0.5 ml, Triiodothyronine 0.5 ml, Epinephrine 0.5 ml and Human Epidermal Growth Factor 0.5 ml. Single quots were added to 500 ml of BEBM (discarding the gentamicin-amphotericin B aliquot)), along with 5 ml of Penicillin - Streptomycin 100X Solution (Gibco®) (5000 units of penicillin and 5000 µg of streptomycin per ml), 500 µl Gentamicin 10 mg/ml (Gibco®) and 2.5 ml fungizone (Gibco®). Solution was mixed by inverting and placed in 50 ml tubes at 4^0C until ready for use.

2.3.3. Primary 50/50 bronchial epithelium media – to aid differentiation

DMEM - Dulbecco's Modified Eagle Medium 250 ml (Gibco®) was added to 250 ml of Bronchial Epithelial Cell Basal Medium (Lonza Clonetics®). To this mixture BEGM® Single Quots Kit® (Lonza Clonetics® which contain Bovine Pituitary Extract 2 ml, Insulin 0.5 ml, Hydrocortisone 0.5 ml, Gentamicin / Amphotericin-1000 0.5 ml, Retinoic Acid 0.5 ml, Transferrin 0.5 ml, Triiodothyronine 0.5 ml, Epinephrine 0.5 ml and Human Epidermal Growth Factor 0.5 ml (discarded the gentamicin-amphotericin B aliquot)) were added, along with 5 ml of Penicillin - Streptomycin 100X Solution (Gibco®) (5000 units of penicillin and 5000 µg of streptomycin per ml), 500 µl Gentamicin 10 mg/ml, (Invitrogen) and 2.5 ml fungizone (Gibco®), 0.5 ml BSA (1.5mg/ml) (Sigma Aldrich®, USA) and 25 µl of retinoic acid (Sigma Aldrich®, USA). The media was filter sterilised and mixed by inverting and placed in 50ml tubes at 4 ^{0}C until ready for use.

2.3.4. Establishing Primary CF bronchial epithelial cell cultures

The bronchial brush with cells attached was transferred to 5ml of BEGM in a 15 ml tube. CF bronchial epithelial cells were agitated from the bronchial brush with a pipette within the 5 ml of BEGM media. Once the cells had been removed from the brush, the brush was discarded and cells were pelleted at 260 g for 5 min at 4^{0}C. The pellet was resuspended in 3-4 ml BEGM Primary media. A T25 (Corning® Flask, USA) flask was rinsed with PBS twice to remove any excess collagen and labelled with sample number, date and Passage number (P0)-1. Cells were incubated at 37^{0}C in 5% CO_2 (Sanyo CO_2 Incubator). The sample number, patient number, date of bronchial brushing and CF mutation (if required) was recorded on a separate data sheet. One day after seeding into the first T25 flask, unattached cells were removed and pelleted. Cells were resuspended in a new T25 flask precoated with collagen as described in section 2.2.3 of materials and methods and placed at 37^{0}C in 5% CO_2 and labelled (P0)-2 with both flasks placed at 37^{0}C in 5% CO_2. On the third day of culture, unattached cells from (P0)-2 were removed and pelleted as before. Cells were then resuspend in 3 ml of BEGM and placed in a new T25 flask labelled (P0)-3. All 3 flasks were placed at 37^{0}C in 5% CO_2. On the fourth day of culture any cells that had not attached to the flask were discarded.

This method allowed for the maximum amount of cells to be retrieved from each bronchial brushing.

2.3.5. Splitting Primary CF bronchial epithelium cells

As in section 2.2.5. of materials and methods with BEGM Primary media replacing BEGM.

2.3.6. Seeding Primary CF bronchial epithelium cells onto inserts

As in section 2.2.6 materials and methods. However, BEGM Primary was supplemented for BEGM. For differentiation of cells Primary 50/50 bronchial epithelium media is used instead of Day 2 media. Primary CF bronchial epithelial cells do not differentiate well in Day 2 media.

2.3.7. Air-liquid interface Culture of Primary CF bronchial epithelial cells

As in section 2.2.8. materials and methods with Primary 50/50 bronchial epithelium media was supplemented for Day 2.

2.3.8. Feeding cells CF bronchial epithelial cells

As described in section 2.2.9. of materials and methods. However, BEGM media is replaced with Primary BEGM media and Day 2 media is replaced with Primary 50/50 bronchial epithelium media.

2.3.9. Ethics committee approval and consent

Ethical approval for the study was obtained from the Ethics (Medical Research) Committee of Our Lady's Children's Hospital, Dublin under The study of Host Immunity and Early Lung Disease in Children with Cystic Fibrosis (SHIELD CF). Full written informed consent was obtained from the parents of patients on the day of bronchoscopy. Primary CF bronchial epithelial cells were obtained from endobronchial biopsies from patients under the age of 6 years who at the time of the BAL procedure are deemed to be fit for the procedure by a paediatrician. Patient number and information privy to this thesis such as sex, age, mutation and previous infections were recorded (see appendix), however children under the age of 6 do not perform lung function tests and therefore FEV1 value cannot be obtained.

2.4. ASL height measurement

Airway Surface Liquid (ASL) height was measured using a live cell fluorescence conflocal microscopy protocol adapted from Tarran *et al* 2006 (47). The ASL height was measured directly by scanning in the vertical XZ plane by confocal microscopy

(Zeiss LSM 700, Germany). The ASL was labelled with Texas Red ® (Invitrogen™) conjugated to 10 –kDa dextran (10 kD, Invitrogen™) which freely difusses in the ASL but is impermeant across the epithelium. Epithelial cells were stained using Calcein Green AM (5 μM, Invitrogen™) as a vital dye used to determine cell viability in eukaryotic cells. To label the ASL, 15 μl of PBS containing 1 mg/ml of dextran Texas Red® was added to the apical aspect of the well-differentiated airway epithelium ~16-18 h prior to image acquistion. Calcein Green – AM was dissolved in culture medium and added to the basolateral compartment of the inserts 1.5 h before image acquisition. Before the acquisition Fluorinet™ electronic fluid Perfluorocarbon 72 (FC-72), (3M, USA) was added to the apical compartment of the insert at a volume of 0.5ml. Perfluorocarbon is immiscible with the ASL and was used to prevent ASL evaporation on transferring the inserts from the incubator to the microscope stage and during acquisitions of images. The epithelium and ASL were imaged in the Z-plane using a Zeiss LSM700 at 40X objective. For average ASL height measurements each Z-stack was measured in 9 different regions in the XZ scanned area using the Zeiss LSM image analyser software (Zeiss, Germany). A mean ASL height value was recorded for each Z-stack.

2.5. Immunocytochemistry

2.5.1. Receptor identification

Immunofluorescence techniques were used to visualise the cellular expression of ALX/FPR2, P2Y2, P2Y11 receptors. NuLi-1, CuFi-1 cell lines and primary CFBE cells were grown in ALI until differentiation had occurred (TEER had reached >700 Ω/cm2 and holding back fluid as described in sections 2.2.9 and 2.3.7. of Materials and Methods). Cells were treated and fixed with paraformaldehyde (4% w/v) (Thermo Scientific, USA) in PBS for 15 min at room temperature (RT). After two washes with PBS cells were permeabilised in Triton-X 100 (0.4% v/v) containing PBS for 10 min and blocked with BSA (3% w/v) in PBS. Cells were incubated for 1-2 h at room temperature with the primary antibody. Cells were then washed three times with PBS and incubated with secondary antibody conjugated to an Alexflour probe for 1 h. Finally cells were washed with PBS three times and mounted with Vectasheild mounting medium (Vector Laboratories, UK) containing DAPI blue overnight at 4^0C. Immunoflourescene images were observed using the Zeiss 700 confoucal microscopy at 60X magnification. Images were analysed using the Zeiss LSM image analyser software.

2.5.2. Visualisation *of P. aeruginosa* invasion

NuLi-1 and CuFi-1 cells were grown in ALI until differentiation had occurred. Cells were infected with *P. aeruginosa* as described in section 6.2.5. of materials and methods. After inoculation of *P. aeruginosa* epithelial cells and bacteria were fixed with paraformaldehyde (4% w/v) (Thermo Scientific, USA) in PBS for 15 min at room temperature (RT). After two washes with PBS, cells were permeabilised in Triton-X 100 (0.4% v/v) containing PBS for 10 min and blocked with goat serum (10% v/v) and BSA (1% w/v) in PBS for 1 h at room temperature. Cells were incubated for 1-2 h at room temperature with the anti-*P.aeruginosa* primary antibody (see section 6.2.5.1. Synthesis of *P.aerginosa* antibody) in blocking solution as above. Cells were then washed and incubated with secondary antibody conjugated to an Alexflour (Invitrogen^{TM}) in blocking solution and probed for 1 h at room temperature. Finally cells were washed three times in PBS and mounted with Vectasheild mounting medium (Vector Laboratories, UK) containing DAPI blue overnight at 4^{0}C. Immunofluorescence images were observed using the Zeiss 700 confoucal microscopy at 60X magnification. Images were analysed using the Zeiss LSM image analyser software.

2.6. Western Blotting

2.6.1. Protein extraction

Proteins were extracted from airway epithelial cells for Western blotting. Cells were grown on 6 well plates or fully differentiated on permeable filters. After the cells were treated under the required specific conditions the plates were put on ice. Each well or filter was then washed three times with 2 ml of cold PBS to remove any culture media. Following the washing, 100 µl of lysis buffer (RIPA Buffer, Sigma-Aldrich®), supplemented with protease inhibitor cocktail (Roche, Switzerland) and a further detergent *N*- lauroylasacrcosine sodium salt (Sigma-Aldrich®, USA) to lyse membrane bound proteins was added for 5 min to each well of a 6 well plate. For cells grown on filters, 50 µl of RIPA and protease inhibitor was added on ice. A cell scraper was then used to scrape all the attached cells from each well or filter. The cell suspension was collected and deposited into a labelled

1.5 ml eppendorf containing detergent *N*- lauroylasacrcosine sodium salt (Sigma-Aldrich®, USA). The sample was vortexed and left on ice for 30 min. The protein suspension was sonicated at an amplitude of 50% for 20sec pulse (Sonics-Vibra Cell^{TM}) and the amount of protein in each sample was determined using BCA kit (Thermo Scientific, USA) as in section 2.6.2. of materials and methods. Following sonification equal amounts of Lammelli buffer (Sigma-Aldrich®, USA) was added to the protein lysate and stored at 4^0C until ready for use. Addition of the lammelli buffer is crucial to limit protein degradation during storage.

2.6.2. Protein quantification

The amount of protein collected for each sample was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA). For one sample 200 µl of reaction mixture was required. Of the protein sample 10 µl was added to a 96 well plate (Corning, USA) in duplicate. Standards were prepared and 10 µl of each standard was added to the plate. To each well used 200 µl of the reaction mixture was added. The plate was then shaken briefly on a plate shaker for 30 sec and placed in at 37^0C incubator for 30 min. The plate was removed from the incubator and left to cool for a couple of min before being inserted into the plate reader (Synergy MX, Bioteck, USA). The plate was read at 562nm. The amount of protein in each sample was determined by a standard curve.

2.6.3. Protein separation by SDS PAGE

Protein samples were prepared so that each sample contained the same concentration of protein. A total volume of 30 µl was required with a volume containing 20 µg of protein. These mixtures were prepared in eppendorfs and then boiled for 10 min (Stuart, Blockheater, SBH130DC, UK). While the samples were boiled, the blotting apparatus (BioRad mini-Protean®) was prepared. To one side of a gasket a gel was placed, to the other side a blank plate was added. Gels were prepared as in Table 2.1. of materials and methods. The gasket was placed in a tank and its seal between the gasket and the gels was checked by adding running buffer (section 2.7.6.4 of materials and methods) into the gasket. Once the samples had boiled for 10 min they were removed and spun down for 30 sec at 500 g in a Eppendorf Minispin and then mixed with a pipette. The samples were then loaded into each lane and position of sample recorded. After the samples were loaded, a protein ladder (Spectra™, Thermo Scientific, USA) was added to the remaining lane. The electrophoresis power pack (BioRad PowerPac™) was set to 100v until the protein had reached the resolving gel. Once the protein had left the stacking gel the power pack was increased to 130v for the desired time.

2.6.4. Transfer of protein

Once the protein migrated down to the bottom of the gel the power pack was turned off and the tank opened up. The gel was then removed from the gasket and placed in the tray filled with transfer buffer made as detailed in section 2.7.6.5. of materials and methods Then in a tray containing a small volume of cold transfer buffer, 2 pieces of wadding and 2 pieces of western blot filter paper (Thermo Scientific, USA) were placed. Next, 2 pieces of polyvinylidene difluoride membrane (PVDF) transfer membrane (Thermo Scientific, USA) were cut to size and activated by placing in methanol (Lennox, IE) for 30 sec. After which they were then transferred to the first container with transfer buffer. The gel was removed from its cast using a pipette to gently wedge either side open. Once the gel was released, a sandwich consisting of plate, sponge, blotting paper, PVDF, gel, blotting paper, sponge and finally another plate was sealed and placed in the appropriate gasket making sure the plates are positioned correctly in the gasket. An ice pack was then placed in the tank to keep the transfer buffer cool. The lid was placed on the tank and the power pack set to at 100v for time determined by the size of the protein of interest.

After this time the apparatus was dissembled and the membrane retrieved. The membrane was then put into a dish containing 10 ml of ponceau red stain (Sigma-Aldrich®) to check for successful protein transfer. After which the blot was washed twice in 5 ml of PBS-Tween (0.01%) (w/v) for 10 min. The blot was placed in 10 ml of blocking buffer (5% w/v dry skimmed milk + PBS) and then placed on a plate shaker for 1 h at RT. Once the blot was blocked it was placed in a square petri dish and 10 ml of primary antibody solution was added and placed on a shaker overnight at 4^0C. Primary antibody solution was made up with 5% (w/v) dry skimmed milk + PBS-Tween (0.01%) (w/v). The following day the blot was washed three times for 15 min with PBS-Tween (0.01%) (w/v). A 10ml solution containing 5% dry skimmed milk + PBS-Tween (0.01%) (w/v) and a secondary antibody linked to horseradish peroxidase was prepared. This solution was poured into a dish containing the blot and put on a plate shaker for 1 h at RT. Finally the blot was washed three times with PBS-Tween (0.01%) (w/v) for 15 min before development.

2.6.5. Blot development

The blot was developed with SuperSignal West Dura Chemiluminescent Substrate (Thermo scientific, USA). The solution from this kit was made by mixing equal parts of both the solutions provided. This mixed solution was then poured on top of the blot. The chemiluminescent was allowed to sit on the blot for 3 min. After which the blot was removed and placed in a cassette and closed. In the dark room an X-ray film (CL-Xpousure^{TM}, Film, Thermo Thermo Scientific, USA) was placed over the blot for differing lengths of time depending on the strength of the signal. The x-ray film was then placed in developer (Kodak Developer, Sigma Aldrich®) until a signal was achieved. The film was washed quickly with water and fixed (Kodak Fixer, Sigma Aldrich®). Once the film was dried an electronic copy was recorded by scanning the film. This scanned image of the blot was used for optical density measurements using Image J software.

2.7.6. Buffers and reagents for SDS-PAGE

2.7.6.1. 1.5M Tris buffer (pH8.8)

181.65 g of Tris Base was dissolved (Sigma-Aldrich®) in 800 ml of dH_20 and adjust pH to 8.8 with concentrated HCL. Bring up to volume of 1 litre with distilled H_2O and store at 4^0C.

2.7.6.2. 0.5M Tris buffer (pH6.8)

181.65 g of Tris Base was dissolved (Sigma-Aldrich®) in 700 mls of distilled H_2O and adjust pH to 6.8 with concentrated HCl (Sigma-Aldrich®) and brought up to a final volume of 1 litre with distilled H_2O and store at 4^0C.

2.7.6.3. 10% Ammonium persulphate (10% APS)

To prepare a 10% (w/v) solution: 1 g ammonium persulfate was dissolved (Sigma-Aldrich®) in 10 mL of H_2O and store at -20^0C

2.7.6.4. Running buffer

1 litre of running buffer was made by measuring 14.0g of glycine (Sigma-Aldrich®), 3.0 g of Tris Base (Sigma-Aldrich®), and 1.0 g of SDS (Sigma-Aldrich®) dissolved in 1 litre of distilled H_2O. The mixture was then mixed and stored at room temperature in the laboratory until ready for use.

2.7.6.5. Transfer buffer

1 litre of transfer buffer was made by mixing 14.1 g of glycine (Sigma-Aldrich®), 3.03 g of Tris Base (Sigma-Aldrich®), 200 mls methanol (Lennox, IE) and dissolved in 800 mls of dH_2O. It was then mixed using a magnetic stirrer before storage at 4^0C overnight.

2.7.6.6. PBS-Tween 0.1%

To 500 ml of DPBS (Gibco®), 500 µl Tween-20 (Sigma-Aldrich®) was added to create a 0.1% tween solution. The solution was stored at room temperature.

2.7.6.7. Ponceau Red

5 ml of glacial acetic acid (Fisher. IE), 90 ml of deionized water and 5 mg (0.1% w/v) Panceau Powder (Sigma-Aldrich®) were added and kept at room temperature until ready for use.

Table 2.1: Concentration and volume of reagents required for SDS-PAGE Gels

RESOVLING GEL	7 ml (1 gel)	14 ml (2 gels)	28 ml (4 gels)
8% Gel			
ddH_2O	3.2 ml	6.5 ml	12.9 ml
30% acrylamide (Sigma-Aldrich®)	1.8 ml	3.7 ml	7.5 ml
1.5M Tris (pH8.8)	1.8 ml	3.5 ml	7 ml
10% (w/v) SDS (Sigma-Aldrich®)	70 µl	140 µl	280 µl
10% (w/v) Ammonium persulphate (Sigma-Aldrich®)	70 µl	140 µl	280 µl
TEMED (Sigma-Aldrich®)	4.2 µl	8.4 µl	16.8 µl
10% Gel			
ddH_2O	2.7 ml	5.6 ml	11.1 ml
30% acrylamide (Sigma-Aldrich®)	2.3 ml	4.7 ml	9.3 ml
1.5M Tris (pH8.8)	1.8 ml	3.5 ml	7 ml
10% (w/v) SDS (Sigma-Aldrich®)	70 µl	140 µl	280 µl
10% (w/v) Ammonium	70 µl	140 µl	280 µl

persulphate (Sigma-Aldrich®)			
TEMED (Sigma-Aldrich®)	2.8 µl	5.6 µl	11.2 µl

STACKING GEL	**1 gel**	**2 gels**	**4 gels**
5% Gel			
ddH_2O	1.4 ml	2.7ml	5.4 ml
30% acrylamide (Sigma-Aldrich®)	330 µl	680 µl	1.4 ml
1.5M Tris (pH8.8)	250 µl	500 µl	1 ml
10% (w/v) SDS (Sigma-Aldrich®)	20 µl	40 µl	80 µl
10% (w/v) Ammonium persulphate (Sigma-Aldrich®)	20 µl	40 µl	80 µl
TEMED (Sigma-Aldrich®)	2 µl	4 µl	8 µl

Table 2.2: List of chemical inhibitors

Inhibitor	Abbreviation	Purpose	Application	Time	Concentration	Reference
BOC-2		inhibitor of ALX/FPR2	Basolateral	15 min pre-treatment	10 µM	(120)
Bumentanide	Bumet	inhibitor of the Na/K/2Cl co-transporter	Basolateral	15 min pre-treatment	1 µM	(47)
10Panx		inhibitor of Pannexin1 channel	Basolateral	30 min pre-treatment	100 µM	(121)
Probenecid	PROB	inhibitor of Pannexin1 channel	Basolateral	15 min pre-treatment	10 µM	(122)
Carbenoxolone	CBX	general inhibitor of Connexin channel	Basolateral	15 min pre-treatment	10 µM	(127)
NF340		inhibitor of P2Y11 receptors	Basolateral	20 min pre-treatment	1 µM/100 M	(123)
H89		inhibitor of PKA	Basolateral	20 min pre-treatment	10 µM	(124)
Alloxazine	Allox	inhibitor of A2b receptr	Basolateral	20 min pre-treatment	10 µM	(63)

RESULTS

Chapter III - Lipoxin A_4 increases airway surface liquid layer height in bronchial epithelium

3.1. Introduction

The hallmark of CF lung disease is chronic infection and inflammation resulting in progressive lung destruction and remodelling of the airways, leading to early death of CF patients. This chronic infection is at least in part related to the ineffective mucociliary clearance found in the CF lung. In normal lungs bacteria and other foreign debris are shuttled away from the lung by the mucociliary escalator which is reliant on the ASL (periciliary liquid and mucus layers) and cilia that transport mucus and foreign particles to the mouth where bacteria is either ingested and destroyed by the stomach or expelled from the body by the mouth. The activity of several types of Cl^- channels, which include the CFTR, drives water transport to generate an ASL, with Na^+ absorption through ENaC stimulating water re-absorption. The mutation in the CFTR gene results in the airway epithelium of CF suffers failing to transport Cl^- and water. This leads to a reduced ASL height and impaired mucocillary clearance resulting in chronic pulmonary disease (48). Hyperabsorption of Na^+ further dehydrates the periciliary layer and compromises cilary beating and mucociliary clearance. Dehydration of the airways favours chronic infection and inflammation that lead to progressive lung disease which is the greatest cause of morbidity and mortality of CF patients. The identification of molecules or biological agents that can increase the ASL height by activating an alternative Cl^- current and/or inhibiting the hyperabsorption of Na^+ is imperative to increasing the life expectancy of the CF patient.

LXA_4 is an endogenous lipid mediator derived from arachidonic acid which is produced at inflammatory sites from the interaction of lipoxygenase activities of several cell types including leukocytes, platelets and epithelial cells. LXA_4 plays a role in resolving the inflammatory process by modulating neutrophilic inflammation, clearing apoptotic PMN and inhibiting the production of pro-inflammatory cytokines (4, 125).

Karp *et al* 2004 have shown that LXA_4 levels are reduced in the lungs of CF patients and this reduction could contribute to the sustained inflammation and infection observed in the lungs of CF patients (3). However, little is understood about the role of LXA_4 in the lungs of CF patients. Reports have shown that bronchial epithelium express the lipoxin receptor, the ALX/FPR2, and activation of this receptor by LXA_4 in the 16HBE14o- cell lines causes intracellular Ca^{2+} mobilisation and Cl^- secretion (126). Intracellular Ca^{2+} is a key regulator of Cl^- transport in epithelial cells. The activation of Cl^- channels in the apical membrane in the airway epithelium leads to efflux of Cl^- into the lumen. This Cl^- efflux provides the electrical potential difference to drive Na^+ with osmotically obliged water across the epithelial tight junctions. The net transepithelial salt and water secretion is the molecular basis for hydration of the airways (51). The loss of Cl^- transport in CF lung epithelium leads to Na^+ hyperabsorption followed by water leading to the chronic dehydration of the airways (50)

3.1.2. Aim

In this chapter, the hypothesis that LXA_4 can increase the ASL height in primary cultures of CF bronchial epithelium and in non-CF and CF bronchial epithelial cell lines was investigated using live-cell imaging by confocal microscopy. LXA_4 is degraded and inactivated quite rapidly by 15-hydroxyprostaglandin dehydrogenase which catalyses the oxidation of LXA_4 by the dehydrogenation at C-15 and oxidation at C-20 (106, 127). Because of this rapid inactivation at the sites of inflammation, stable analogues of LXA_4 have been developed to withstand this degradation. One such stable analogue is the TA39 molecule which was developed by *Guiry et al (128)*. The physiological effect of TA39 on ASL dynamics was also investigated in CF and non-CF airway epithelia.

3.2. Specific Methodologies

3.2.1. Cell culture

NuLi-1, CuFi-1 and CuFi-3 cells were all grown to fully differentiated well polarised epithelium as previously described in section 2.2. of the materials and methods. Primary CF bronchial epithelial cells were obtained from endobronchial biopsies from patients under the age of 6 years (patient numbers HCFBE68, HCFBE69 and HCFBE76; see appendix). CF bronchial epithelial cells were grown and cultured as described in section 2.3. of materials and methods.

3.2.2. ASL height measurements

ASL height measurements were recorded from fully differentiated epithelial cells using live-cell confocal fluorescence microscopy from an adapted protocol of Tarrren *et al* 2006 *(47)*. The method of ASL height measurements is outlined in section 2.4. of materials and methods. In short, ASL was labelled with 15 µl of Texas red®-dextran overnight. Epithelial cells were stained with Calcein-AM at 37^0C for 1.5 h prior to acquisition. Fluorinet^{TM} electronic fluid Perflurocarbon-72 was added to the apical compartment of the insert and at a volume of 0.5 ml. The ASL height was recorded with Zeiss LSM 710 using the 40x objective and measured using the Zeiss LSM Image analyser software.

3.2.3. Data analysis

ASL height measurements obtained from 3-4 confocal image acquisitions and nine regions of interest were measured. Values for each insert were averaged and the mean value obtained. The n number refers to the number of inserts used. NuLi-1, CuFi-1 and CuFi-3 experiments were repeated on 3 different cell passages under the same conditions. Data is presented as mean ± S.E.M. of n experiments. Statistical significance was obtained using the one way ANOVA test. Comparisons within groups were made using the post hoc test, Newman-Keuls. In all tests a *P* value ≤ 0.05 was deemed significant. All statistical analyses were carried out by GraphPad Prism.

3.3. Results

3.3.1 LXA_4 increases ASL height in NuLi-1 and CuFi-1 cells

ASL height is crucial for adequate mucociliary clearance of the lungs in CF patients. ASL was visualised using Dextran Texas red and ASL height on NuLi-1 (non-CF) and CuFi-1(CF) cells was analysed using confocal microscopy. NuLi-1 cells treated with LXA_4 (1 nM) resulted in a significant increase in ASL height 15 min (n= 6, $P \leq 0.001$, ANOVA), 30 min (n= 6, $P \leq 0.001$, ANOVA) and 45 min (n= 6, $P \leq 0.001$, ANOVA) post stimualtion compared to basal levels (Figure 3.1, (A)). NuLi-1 cells are a fully functioning airway model that can quickly mediate the increase in ASL height back to normal levels (~ 7 µM) 1 h post stimulation with LXA_4. This return to basal levels is continued at 24 h and 48 h. In comparison the CF cell line CuFi-1 observed a significant increase in ASL height for 15 min (n= 6, $P \leq 0.001$, ANOVA), 30 min (n= 6, $P \leq 0.001$, ANOVA), 45 min (n= 6, $P \leq 0.001$, ANOVA), 1 h (n= 6, $P \leq 0.001$, ANOVA) and 24 h (n= 6, $P \leq 0.001$, ANOVA) with LXA_4 post-stimulation with a return to basal levels at 48 h. The increase in ASL height induced by LXA_4 in CuFi-1 cells is above 7 µM which is considered the minimum height required for an effective mucociliary clearance (Figure 3.1, (B)). These results suggest that LXA_4 could play a pivotal role in CF bronchial epithelium by increasing ASL height and creating an effective mucocillary clearance which may elevate the symptoms observed in the CF lung.

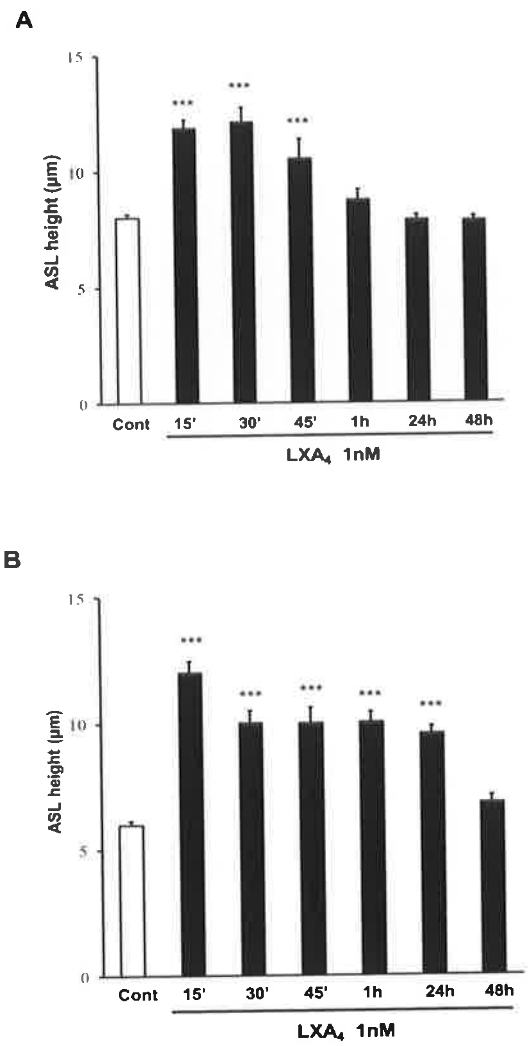


Figure 3.1: LXA_4 increases ASL height in NuLi-1 and CuFi-1 cell lines. (**A**) ASL height was measured from non-CF (NuLi-1) cells using Dextran Texas Red. LXA_4 significantly increased ASL height 15, 30 and 45 minutes post stimulation (n=6, $P \leq 0.001$, ANOVA). 1 h post stimulation resulted in an increase in ASL height but did not reach significance. 24 h and 48 h post inoculation ASL height in NuLi-1 cells returned to basal levels. (**B**) CuFi-1 (CF) cell lines ASL height was significantly increased 15 min, 30 min, 45 min, 1 h and 24 h post stimulation with LXA_4 (n=6, $P \leq 0.001$, ANOVA). 48 h post stimulation ASL height returned to basal levels in CuFi-1 cells.

3.3.2. ASL height increase visualized by live cell imaging

The increase in ASL height induced by LXA_4 was observed and imaged using the Zeiss 710 confocal microscope. Control conditions of NuLi-1 cells stained with calcein green, show a continuous ASL with height and volume across the apical surface of the epithelium which has been stained with Dextran Texas Red. When NuLi-1 cells were stimulated with LXA_4 (1 nM) there was an increase in the volume, height and intensity of the ASL fluorescence (Figure 3.2, (A)). Live cell images of CuFi-1 cells (CF) show a thin broken and disrupted ASL in control conditions. Upon stimulation with LXA_4 and increase in ASL height and volume was observed across the epithelium surface (green) 15 min post stimulation (Figure 3.2, (B)).

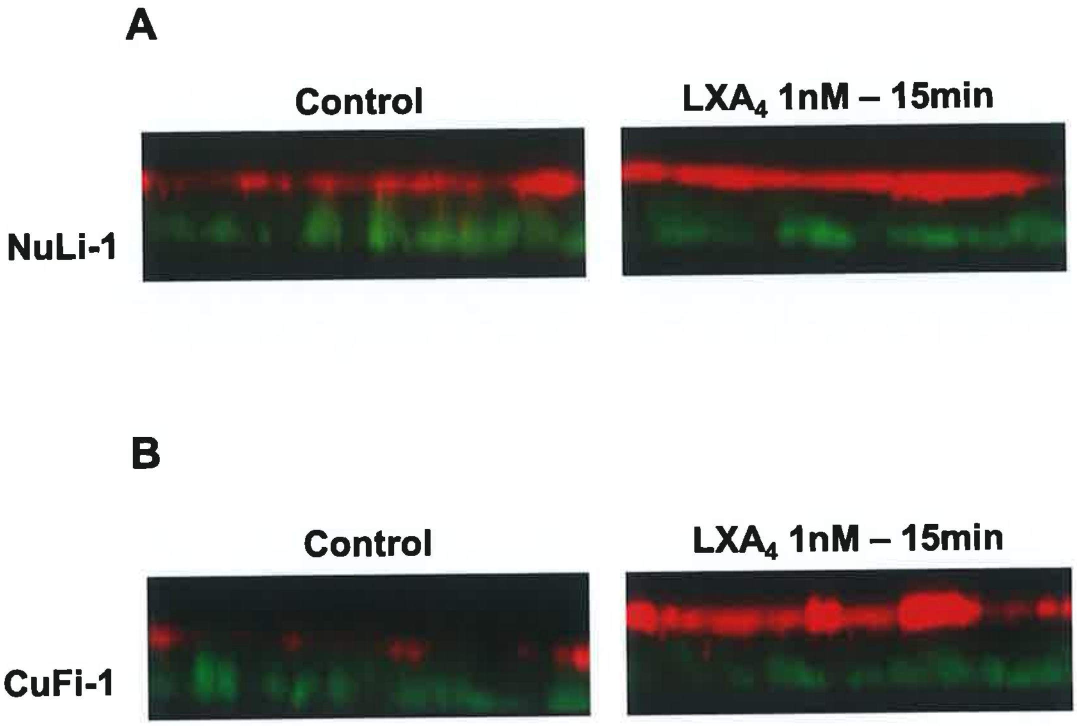


Figure 3.2: ASL height increase induced by LXA_4 visualised by live cell imaging of NuLi-1 and CuFi-1 cells. ASL is stained red by Dextran Texas Red and epithelial cells were stained green with Calcien Green. Live cell images were taken by confocal microscopy. (**A**) Typical image of NuLi-1 cells at control conditions and treated with LXA_4 (1nM). NuLi-1 cells stimulated with LXA_4 show an increase in ASL height and volume across the epithelial apical surface. (**B**) The ASL is thin, broken and disrupted in non-stimulated CuFi-1 cells when compared to NuLi-1 cells. There was also an increase in ASL height and volume observed in CuFi-1 cells when stimulated with LXA_4.

3.2.3. LXA_4 increases ASL height in primary CF bronchial epithelial cells

Reconstituted epithelium from bronchial biopsies where treated with LXA_4 (1 nM) and ASL height was measured. ASL height measurements were carried out on 2 different patients samples both homozygous for the ΔF508 mutations. Stimulation with LXA_4 (1 nM) increased ASL height from basal levels (5.49 μM ± 0.07, n=4) to 9.20 μM ± 0.13 (n=4) 15 min post stimulation and to 60 min (7.15 μM ± 0.13, n=4, post stimulation. There was an increase in ASL height compared to basal levels 24 h post stimulation. ASL height returned to basal levels 48 h post stimulation (Figure 3.3, (A)). The increase in ASL height by LXA_4 in primary CF bronchial epithelial cells was imaged using confocal microscopy. 3D image is presented to illustrate a complete visualisation of the scanned epithelium (Figure 3.3, (B)).Typical images of non-stimulated CF bronchial epithelial cells show a thin, broken and disrupted ASL compared to CF bronchial epithelial cells treated with LXA_4 were the ASL is continuous across the epithelium with increased height and volume. Thus LXA_4 can increase ASL height in CF bronchial epithelial cells indicating a possible novel role for LXA_4 in the treatment of CF.

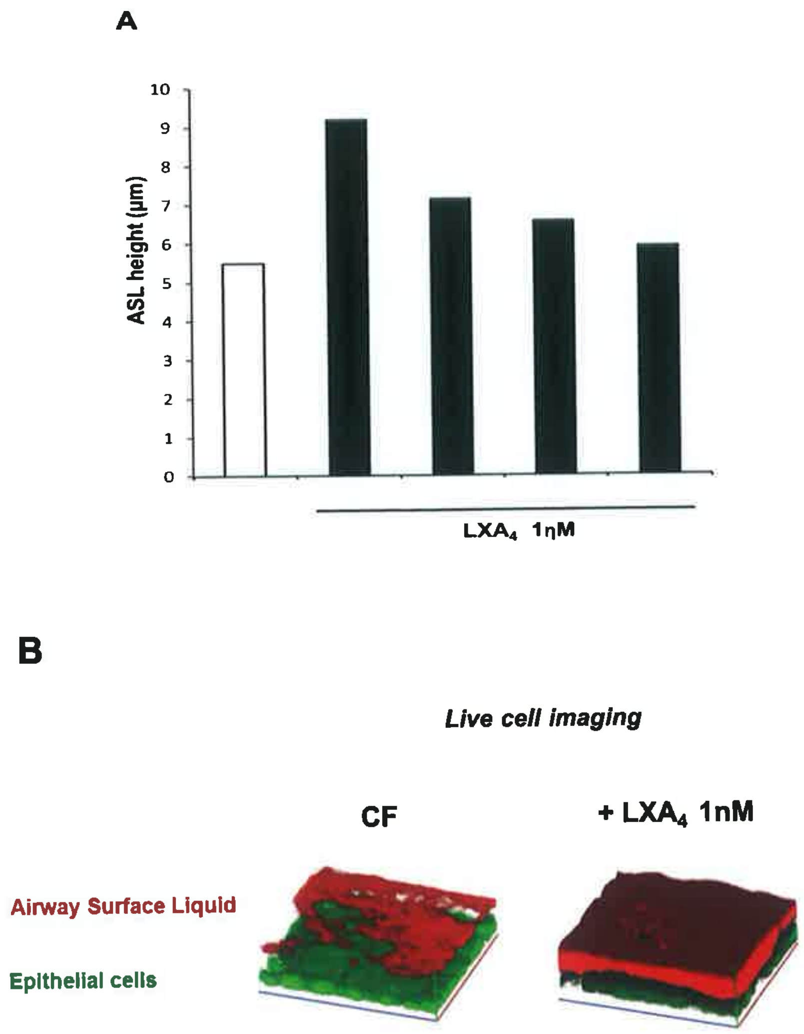


Figure 3.3: ASL height increase is induced by LXA_4 in primary CF bronchial epithelial cells. (**A**) An increase in ASL height in primary CF bronchial epithelial cells was observed 15 min and 60 min post stimulation with LXA_4 (1 nM). 24 h post stimulation also resulted in an increase in ASL height which returned to basal levels at 48 h post stimulation (A). (**B**) Typical 3D image taken from confocal microscopy of the ASL height increase in primary CF bronchial epithelial cells induced by LXA_4.

3.3.4. LXA_4 stable analogue TA39 increases ASL height in NuLi-1 cells

LXA_4 is created at the site of inflammation where it signals for the resolution of inflammation. Endogenous LXA_4 is rapidly degraded and inactivated (4, 127). In our experiments, the exogenous stable analogue of LXA_4, TA39 was employed and the ability to increase ASL height in bronchial epithelium was examined. Differing concentrations of TA39 were used in measuring the effect on ASL height in normal bronchial epithelium. In NuLi-1 cells the TA39 (1 pM) did not significantly increase ASL height at 15min, 60min, 24h and 48h post stimulation compared to basal levels (Figure 3.4, (A)). TA39 (1 nM) mimicked the increase in ASL height induced by LXA_4 (10.31 µM ± 0.15, n=4, ANOVA) at 15min (10.16 µM ± 0.15, n=6, ANOVA) and 60min (9.7 16 µM ± 0.13, n=6, ANOVA) (P *** ≤ 0.001) post stimulation with ASL height returning to basal levels 48 h post stimulation (Figure 3.4, (B)). The increase in ASL height stimulated by TA39 was captured by confocal microscopy 15 min post stimulation (Figure 3.4, (C)).

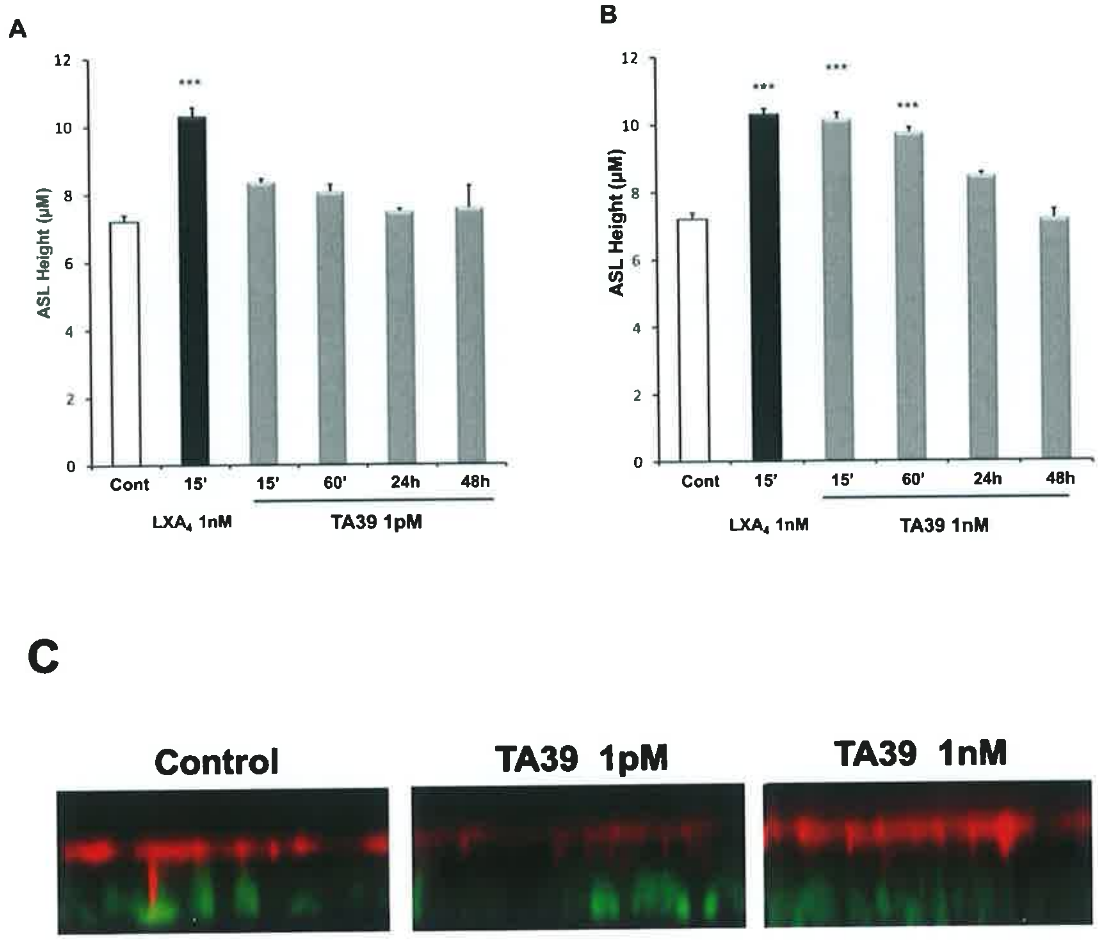


Figure 3.4: LXA_4 stable analogue TA39 increases ASL height in NuLi-1 cells. (**A**) TA39 (1 pM) the stable analogue of LXA_4 did not significantly increase ASL height in NuLi-1 cells. (**B**) NuLi-1 cells treated with TA39 (1nM) mimicked the ASL height increase induced by LXA_4 (1nM) 15 min and 60 min post stimulation (*P* *** ≤ 0.001, n=4, ANOVA). (**C**) Live cell imaging of the ASL was captured by confocal microscopy of NuLi-1 cells treated with 1 nM and 1 pM TA39.

3.3.5. TA39 increases ASL height in CuFi-1 cells

Restoring adequate mucociliary clearance by increasing ASL height is crucial for CF suffers. Therefore natural or synthetic molecules that can increase ASL height in the CF lungs are required that may increase the life expectancy of the CF patient. The stable analogue of LXA_4, TA39, was tested in the CuFi-1 cell line at two different concentrations 1 nM and 1 pM. TA39 (1 pM) significantly increased ASL height from basal levels (5.79 µM ± 0.09, n=4, ANOVA) to 15 min (7.98 µM ± 0.09, n=4, ANOVA), 60 min (8.15 µM ± 0.12, n=4, ANOVA) and 24 h (7.06 µM ± 0.12, n=4, ANOVA) (P *** ≤ 0.001) post stimulation. The ASL returned to basal levels 48 h post stimulation with TA39 (1 pM) (Figure 3.5, (A)). A significant increase is seen in ASL height when stimulated with TA39 (1 nM) 15 min (7.26 µM ± 0.10, n=4, ANOVA), 60 min (7.71 µM ± 0.12, n=4, ANOVA) and 24 h (6.93 µM ± 0.08, n=6, ANOVA) (P *** ≤ 0.001) post stimulation. 48 h post stimulation with TA39 (1 pM) the ASL height returned to basal level (Figure 3.5, (B)). Observations by confocal microscopy of the ASL height induced by the TA39 (1 pM and 1 nM) in CuFi-1 cells is shown in Figure 3.5, 15 min post stimulation (C). The ability of TA39 to mimic LXA_4's ability to increase ASL height in CF bronchial cells can have important implications for a more sustained physiological effect on ASL height in CF lungs. Further testing of the molecule TA39 is required but these preliminary results indicate that TA39 could possibly be implicated in a new therapeutic avenue for the CF patient.

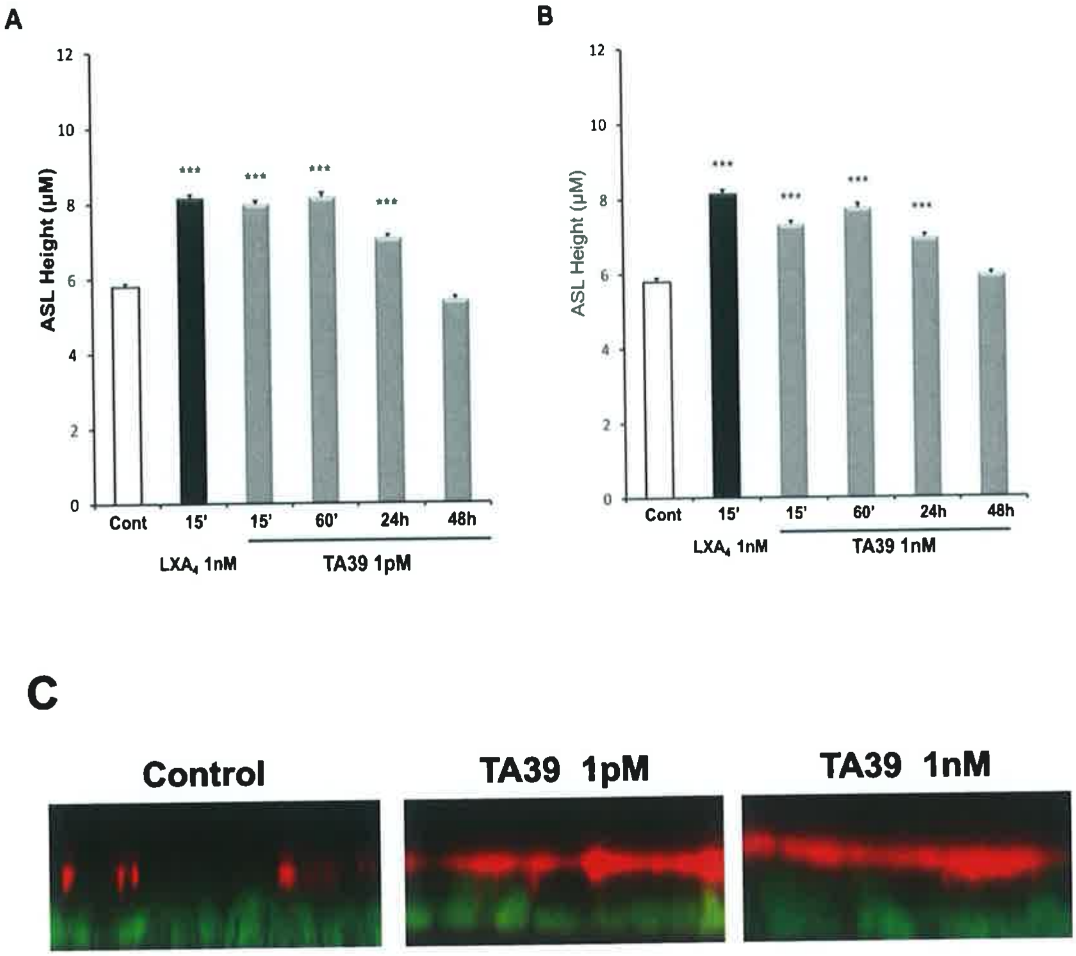


Figure 3.5: TA39 increases ASL height in CuFi-1 cells. TA39 (1 pM) increases ASL height in CuFi-1 cells (**A**) mimicking the increase induced by LXA_4 (1 nM) 15 min post stimulation. The increase in ASL is sustained up to 24 h post stimulation with TA39 (P *** ≤ 0.001, n=4, ANOVA). (**B**) CuFi-1 cells treated with TA39 (1 nM) also resulted in a significant increase in ASL height compared to control conditions which was sustained from 15 min to 24 h post- stimulation (P *** ≤ 0.001, ANOVA)(**C**) The ASL height induced by TA39 was observed by confocal microscopy and images analysed. Live cell images of CuFI-1 cells when stimulated with 1 nM and 1 pM TA39 compared to control conditions.

3.3.6. The increase in ASL height induced by LXA_4 is mediated by ALX/FPR2 receptor and Cl^- secretion

The ALX/FPR2 receptor was investigated as the mediator of the ASL increase induced by LXA_4. Inhibition of ALX/FPR2 receptor in non-CF (NuLi-1) and CF cell lines and CF bronchial epithelial primary cultures (CuFi-3, CuFi-1 and CFBE cells) were tested by the non-specific receptor antagonist Boc-2. It has previously been shown that LXA_4 increases Ca^{2+} mobilization in bronchial epithelium cells (126). NuLi-1 cells stimulated with LXA_4 (1 nM) resulted in an increase in ASL height, this increase was abolished when the ALX/FPR2 receptor was blocked by pre-treatment of Boc-2 (10 μM) 15 min before stimulation with LXA_4. The decrease in ASL height was also observed in CuFi-3, CuFi-1 and primary CF bronchial epithelial cells (Figure 3.6, (A)) when pre-treated with Boc-2.

Bumetanide an inhibitor of the Na/K/2Cl co-transporter was used to investigate the contribution of Cl^- secretion to the production of the ASL. Pre-treatment with Bumetanide (1 μM) for 15 min significantly decreased the basal ASL height (control 7.25 ± 0.07 μm, bumetanide 5.9 ± 0.1 μm (*P* <0.001, n = 6) in NuLi-1 monolayers but had no significant effect on ASL height in CuFi-1, CuFi-3 and CFBE cells (n=6, n=6, n=8) indicating that Cl^- secretion contributes to the generation of the basal ASL height in the non-CF epithelium. Bumetanide (1 μM) significantly decreased the ASL height induced by LXA_4 in cell types tested. The ASL height measured after LXA_4 exposure in the presence of bumetanide was significantly decreased in NuLi-1 (5.6 ± 0.11 μm, n = 5, *P* <0.05, ANOVA), in CuFi-1 (4.86 ± 0.1 μm, n = 6, *P* <0.001, ANOVA) and in CuFi-3 (6.2 ± 0.1 μm, n = 6, *P* <0.001, ANOVA) cell lines and in CF bronchial epithelium primary cultures (4.7 ± 0.2 μm, n = 9, *P* <0.001, ANONVA) compared to LXA_4 only (Figure 3.6, (B-D)).

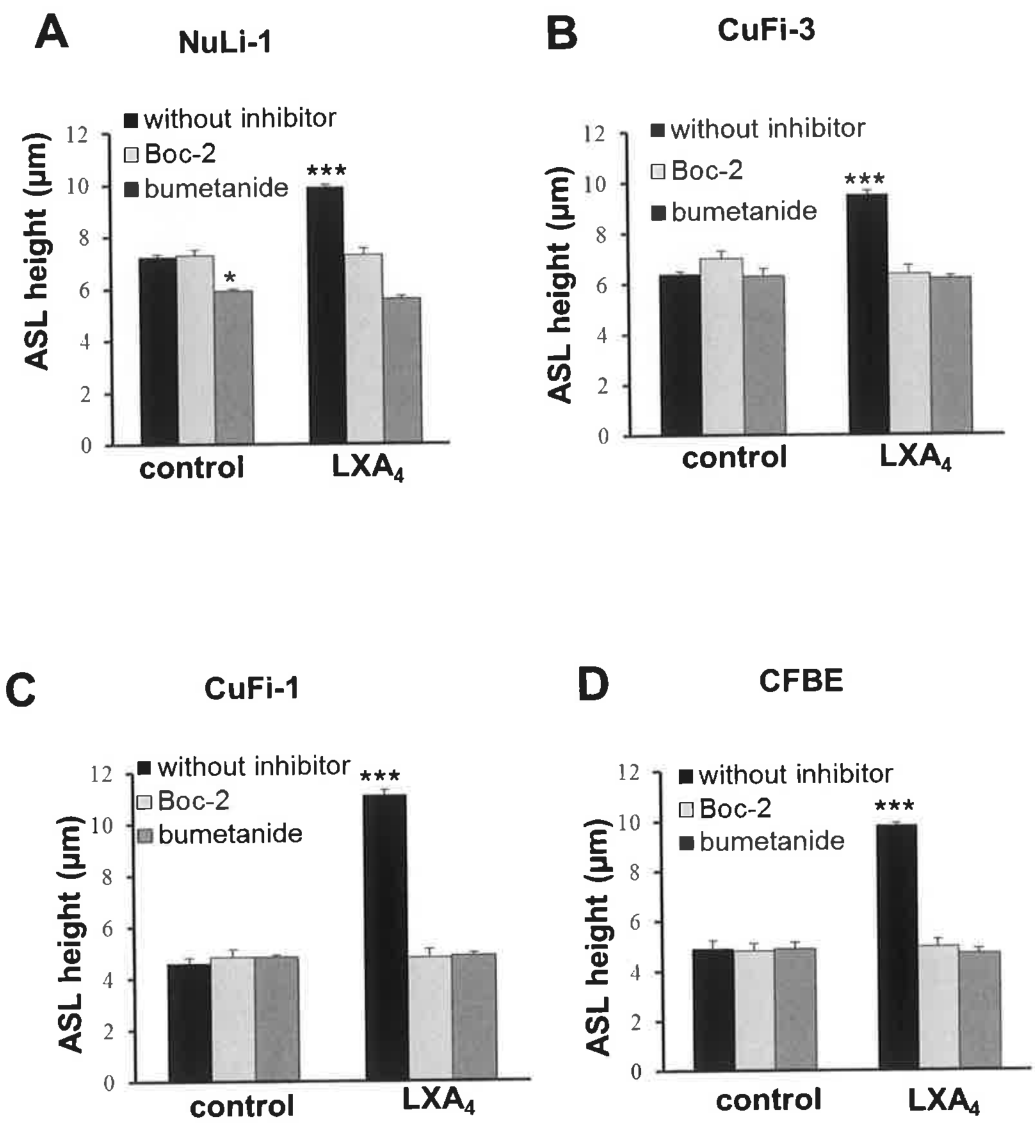


Figure 3.6: LXA_4 induced ASL height increase is mediated by ALX/FPR2 receptor. Blocking LXA_4's receptor, the ALX/FPR2 receptor with Boc-2 (10µM) in NuLi-1 (A), CuFi-3 (B), CuFi-1 (C) and CF bronchial epithelium cells (CFBE) (D) cells resulted in a inhibition of ASL height increase generated by LXA_4. The increase in ASL height created by LXA_4 was also prevented when Ca^{2+} dependent mobilisation of Cl^- was inhibited by pre-treatment with bumetanide (1 µM) NuLi-1, CuFi-3, CuFi-1 and primary CFBE cells. (* $P \leq 0.05$, *** $P \leq 0.001$, ANOVA)

3.3.7. Boc-2 abolishes the effect of TA39 on ASL height increase in NuLi-1 and CuFi-1 cells

Boc-2 abolished the ASL height increase induced by LXA_4 in NuLi-1, CuFi-1 and CF bronchial epithelial cells. TA39 the stable analogue of LXA_4 mimicked the effect on ASL height in NuLi-1 and CuFi-1 cells at a concentration of 1 nM. Inhibition of the ASL height increase by Boc-2, was investigated when NuLi-1 and CuFi-1 cells were stimulated with TA39 (1 nM). NuLi-1 cells pretreated with Boc-2 before stimulation with TA39 (1 nM) resulted in a significant decrease in ASL height generation induced by TA39. Boc-2 alone had no effect on basal levels of ASL height (Figure 3.7, (A)). This decrease in ASL height was also observed in CuFi-1 cells when the ALX/FPR2 receptor was inhibited by Boc-2 (Figure 3.7, (B)). These results provide further evidence that the ASL height increases generated by LXA_4 and its stable analogues involve the ALX/FPR2 receptor.

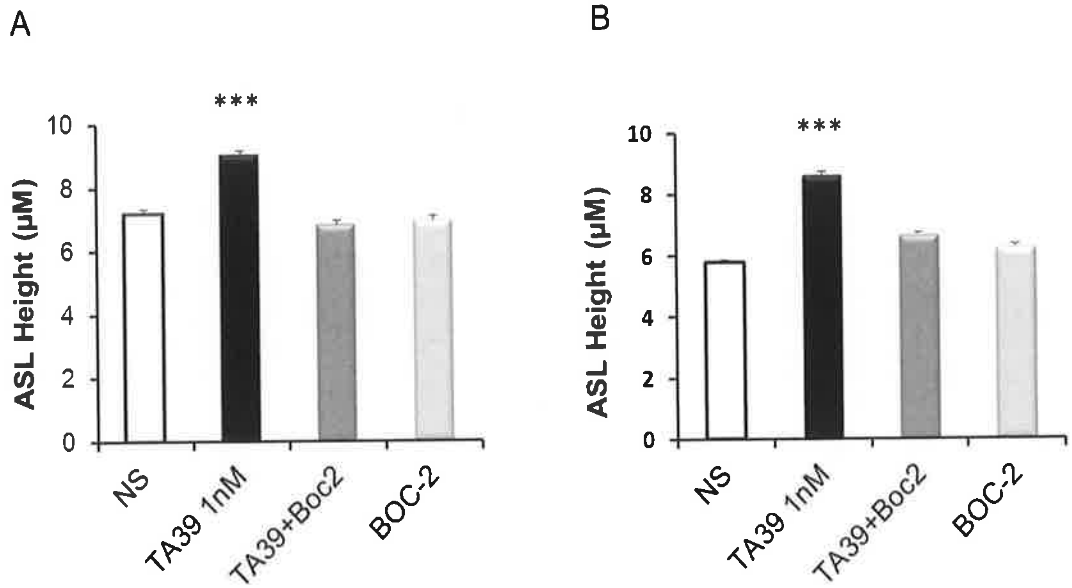


Figure 3.7: Boc-2 inhibits the ASL height increase induced by TA39. (**A**) TA39 (1nM) significantly increased the ASL height in NuLi-1 cells (*P* *** ≤ 0.001, n=4, ANOVA) compared to non-stimulated (NS) conditions. (**B**) The ASL height increase generated by TA39 was also observed on CuFi-1 cells (*P* *** ≤ 0.001, n=4, ANOVA) compared to NS cells. Inhibition of the FPR2/ALX receptor by Boc-2 completely abolished the ASL height increase induced by TA39 in both NuLi-1 and CuFi-1 bronchial epithelial cells.

3.4. Discussion

In the non-CF lung, the ASL forms a thin layer of fluid on the surface of bronchial epithelium which allows cilia to beat effectively. Maintenance of an optimal ASL height for ciliary beat is crucial for the efficacy of mucociliary clearance. Airway surface liquid dehydration leads to mucus plugging resulting in chronic bacterial infection, persistent inflammation and progressive destruction of the lung (43, 129, 130). Bronchial epithelial ion transport regulates the ASL height. In CF, the lack of functional CFTR leads to a reduced ASL height, resulting in an impaired mucociliary clearance that promotes chronic bacterial infection of the airways (50).

CF bronchial epithelia generate a thinner ASL layer than non-CF airway epithelia which is consistent with the reduced ASL in CF airways reported in the literature (48, 49, 54, 131). The non-CF epithelial monolayers showed a continuous ASL layer whereas in CuFi-1 cell lines and CF bronchial epithelium primary cultures the liquid layer was disrupted. Following LXA_4 exposure in CF bronchial epithelia, the ASL height significantly increased in height and volume across the apical membrane.

Inhibition of the ALX/FPR2 receptor by Boc-2 (ALX/FPR2 receptor antagonist) abolished the ASL height induced by LXA_4 in non-CF and CF bronchial epithelium. This result supports the role for ALX/FPR2 receptor in mediating the physiological effects of LXA_4 which has been previously shown in a number of different cell types

(128). The inhibition of ASL height by Boc-2 when cells were pre-treated with TA39 supports the hypothesis that LXA_4's physiological effect on ASL height is mediated by this promiscuous receptor. To further support the role of ALX/FPR2 in the physiological effects induced by LXA_4 our laboratory has obtained preliminary results indicating that upon stimulation with LXA_4 the ALX/FPR2 receptor is rapidly shuttled to the apical membrane. Studies have shown the inability of CFTR inh-172 on the LXA_4 stimulated whole-cell currents indicates that the effect of LXA_4 on Cl^- secretion is not mediated by CFTR activation (129). The fact that bumetanide reduces further the ASL height compared to Boc-2 treatment in NuLi-1 cells but not in CuFi-1 or CuFi-3 cells where functional CFTR is absent reinforces this conclusion. If LXA_4 activated CFTR and Ca^{2+}-dependent Cl^- channels together then matching inhibition of ASL height by Boc-2 and bumetanide in NuLi-1 cells would be expected.

The inhibitory effect of bumetanide indicates that the effect of LXA_4 on ASL height is mainly dependent on stimulation of transpithelial Cl^- transport. However, it cannot be omitted that LXA_4 can also exert its action to increase ASL height through the inhibition of ENaC activity which is known to be stimulated in CF airways and down-regulated by increased intracellular Ca^{2+}.

LXA_4 is readily degraded by oxidation at C-15 or reduction of the C13−C14 double bond and are also subject to ω oxidation at C20. This degradation represents a major problem for the application of lipoxins to be used as therapeutic molecules. Hence compounds of LXA_4 that are biologically and metabolically stable are required for the possible treatment options. Earlier work has shown that native LXA_4 has a maximal effect at 100 nM (132). The increase in ASL height observed in both NuLi-1 and CuFi-1 cells when treated with TA39 (1 nM) mimicked the ASL height increase induced by LXA_4. LXA_4 stable analogues has been tested on a number of cell types and have demonstrated the same physiological effects of native LXA_4.For example, THP-1 cells resulted in an increase in apoptotic human PMNs, monocytes increased adhesion and a decrease in neutrophil accumulation was observed when all were stimulated with stable analogues of LXA_4 (108). The effect of stable analogues of LXA_4 on animal models have also been reported with analogues having an equipotent effect of native LXA_4 (128, 133) . With this in mind further analysis of the TA39 on CF bronchial epithelium should be merited as a potential therapeutic avenue for CF suffers.

This result chapter suggests that LXA_4 and its stable analogues increase ASL height in CF bronchial epithelium and could play a role in a new therapeutic strategy to rehydrate the airways of CF patients. This increase in ASL is determined by the increase in Ca^{2+} mobilisation activating a CaCC on the apical membrane which can bypass the defective CFTR. This increase in ASL height will increase the effectiveness of the mucociliary process which is reduced in the CF lung.

Chapter IV - Activation of P2Y receptors by ATP release induced by lipoxin A_4 restores airway surface liquid height in cystic fibrosis bronchial epithelium

4.1. Introduction

In CF, the airway surface liquid (ASL) is dehydrated and its volume reduced as a result of impaired ion transport. This reduction in ASL height results in an impaired mucociliary clearance leading to chronic bacterial infection, persistent inflammation and a progressive destruction of the lung (10).

As previously described LXA_4 is a biologically active eicosanoid derived from arachidonic acid through lipoxygenases interaction and has been proposed as a novel regulator of adaptive immunity which may have therapeutic potential in chronic immune disorders (101). It has been reported that LXA_4 targets airway epithelium inducing several physiological responses including intracellular Ca^{2+} mobilization and enhanced Cl^- secretion, epithelial repair and tight-junction formation (120, 126, 129, 134) and findings presented in this thesis also report that LXA_4 stimulates an increase in ASL height in both non-CF and CF airway epithelia grown in an air-liquid interface as described in Chapter III - Lipoxin A_4 increases airway surface liquid layer height in bronchial epithelium (135).

LXA_4 is a ligand for the ALX/FPR2 receptor. Studies using Boc-2 an inhibitor of the ALX/FPR2 receptor shows that ALX/FPR2 receptor plays a role in mediating the anti-inflammatory effect caused by LXA_4 (136). In Chapter III, it was reported that the ASL height induced by LXA_4 was abolished by inhibition of the ALX/FPR2 receptor. This result naturally raises the question of how the binding of LXA_4 to the ALX/FPR2 receptor mediates an increase in ASL height, a question that which will be investigated in this chapter.

Reports that airway epithelial cells release ATP and the functional expression of purinergic receptors (P2 receptors) in airway epithelia suggest that the release of nucleotides may control epithelial functions including ion transport, ASL volume homeostasis andepithelial structural integrity (59, 64, 137, 138). Stimulation of airway epithelial Cl^- secretion and inhibition of Na^+ absorption by extracellular nucleotides has been well documented. These nucleotide effects are mediated by P2 receptors expressed in respiratory epithelium (59, 61, 64, 137, 138). The stimulation of Cl^- secretion in CF airways by extracellular nucleotides has triggered an interest in targeting purinoreceptors for CF therapy and in particular the P2Y receptors (59). P2Y receptors are coupled to the Gq protein receptor and trigger cytosolic calcium signal transduction pathways to regulate ion transport in human airway epithelial cell model systems (139). The P2Y11 receptor is unique among P2Y receptors because its acts through both Gq and Gs protein receptors to stimulate both calcium and cAMP signalling pathways (140). Receptors for adenosine, the final product of ATP hydrolysis, also stimulate Cl^- secretion in airway epithelial model systems (61). The major adenosine receptor, the A_2b receptor, activates the cAMP/ PKA signal transduction cascade and eventually CFTR (62).

4.1.2. Aim

In this chapter, the cellular pathways involved in ASL height increase induced by LXA_4 were examined in non-CF and CF bronchial epithelium. We tested the hypothesis that LXA_4 stimulates an apical ATP release from non-CF and CF bronchial epithelium, activating a P2Y pathway as demonstrated in Figure 4.1.

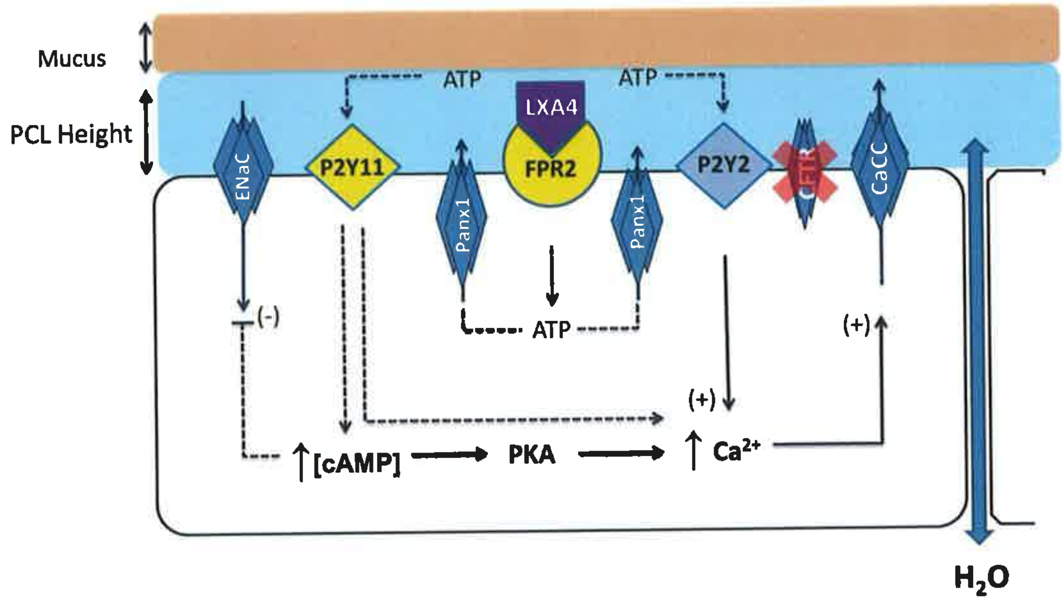


Figure 4.1: Proposed mechanism behind the ASL height increase in bronchial epithelial cells induced by LXA_4. Stimulation of the FPR2 receptor by LXA_4 induces an apical ATP release through the pannexin channels (Panx1) channel activating a purinoreceptor pathway. Activation of the P2Y2 and P2Y11 receptors inhibit the epithelial sodium channel (ENaC) and sodium reabsorption while stimulating chloride secretion out of the cell by calcium activated chloride channels (CaCC), activated through an intracellular calcium (Ca^{2+}) release, which may result in a restored ASL height in CF bronchial epithelial cells.

4.2. Specific Methodologies

4.2.1. ASL height measurements

As in section 3.2.2 of specific methodologies chapter III.

4.2.2. Extracellular ATP measurements

ATP release assays were conducted at room temperature (20-22 ^{0}C) on an anti-vibration table (Newport). Epithelia grown on inserts were washed apically and basolateraly three times with PBS before experiments began. The content of ATP released by epithelial cells was measured by using a commercially available ATP determination kit (A22066 Molecular Probes, Oregan, USA). The bioluminescence assay is based on the reaction of ATP with recombinant firefly luciferase and its substrate luciferin. In short, a standard curve was generated in triplicate in a blackened 96 well plate. Luminescence was read on a Synergy Mx Biotek plate reader (Biotek Instruments, VT, USA) using the Gen5 software program. Unknown ATP containing samples were substituted for ATP standards and read in triplicate. ATP concentration was calculated from a standard calibration curve. The ATP detection limit was 0.1 pM of ATP.

4.2.3. Concentration of cAMP

cAMP was measured using a cAMP Enzyme Immunoassay Kit (Sigma, St Louis, USA) as per kit instructions.

4.2.4. P2Y receptor detection

Using the Immunocytochemistry protocol described in section 2.5.1. of materials and methods, the P2Y11 receptor was identified using rabbit anti-P2Y11 (1:500) primary antibody (Alamone Labs, Israel) in BSA (3% w/v) with PBS. Following 3 washes in PBS. The epithelia were incubated for 1 h with the secondary antibody; AlexaFluor 488-conjugated anti-rabbit (Invitrogen, Auckland, New Zealand) which was diluted 1:2000 in BSA (3% w/v) with PBS at room temperature. The cells were washed three times in PBS and mounted in Vectashield mountant (Vector Laboratories, Peterborough, United Kingdom) containing DAPI overnight at 4^0C . For P2Y2 identification rabbit anti-P2Y2 primary antibody (Abcam, UK) was diluted 1:500 in BSA (3% w/v) with PBS. AlexaFluor 488-conjugated anti-Rabbit was used as a secondary antibody diluted 1:2000 BSA (3% w/v) with PBS. Cells were washed three times in PBS and mounted in Vectashield mountant containing DAPI overnight at 4^0C. The immunofluorescence intensity was measured in 5 regions of interest chosen randomly at the epithelial apex and averaged. The Alexafluor 488-labelled anti-rabbit antibody was visualised at 488 nm excitation wavelength in the 505-530 nm emission range. DAPI was visualised using a 364 nm excitation wavelength and 385-470 nm detection range.

For Western Blotting (see section 2.7. of materials and methods for description of thechnique), rabbit anti-P2Y11 primary antibody (Abcam, UK) (1:500) and rabbit anti-P2Y2 primary antibody (Abcam, UK) (1:400) in 5% milk PBST (0.01% w/v) was used for detection of the protein. Anti-rabbit HRP (1:5000) in 5% milk PBST (0.01% w/v) was used as a secondary antibody (Cell Signalling, Danvers, MA).

Mouse anti-α-tubulin (1:5000) in 5% milk PBST (0.01%) was used as loading controls with anti-mouse HRP (1:5000) in 5% milk PBST (0.01%) as a secondary antibody. Optical density of the immune bands was determined by Image J software.

4.2.5. FPR2 receptor expression

Using the Western Blotting protocol described in section 2.6. of materials and methods, rabbit anti-FPR2 primary antibody (Abcam, UK) (1:2000) in 5% milk PBST (0.01% w/v) was used for protein detection. Anti-rabbit HRP (1:5000) in 5% milk PBST (0.01% w/v) was used as a secondary antibody (Cell Signalling, Danvers, MA). For loading controls mouse α-tubulin (1:5000) in 5% milk PBST (0.01% w/v) was used. Optical density of the immune bands was determined by Image J software.

4.2.6. Data analysis

N number refers to the number of inserts used. NuLi-1 and CuFi-1 experiments were repeated on 3 different cell passages under the same conditions. Primary bronchial cells were obtained from children under 6 years of age (patient numbers HCFBE68, HCFBE69 and HCFBE90, HCFBE107 ; see appendix). Data are presented as mean ± S.E.M. of n experiments. Statistical significance was obtained using the one way ANOVA test. Comparisons within groups were made using the post hoc test, Newman-Keuls. In all tests a P value ≤ 0.05 was deemed significant. All statistical analyses were carried out by GraphPad Prism.

4.3 Results

4.3.1. LXA_4 stimulation of ATP release

The release of ATP from non-CF (NuLi-1) and CF (CuFi-1) airway epithelial cell lines grown under an air-liquid interface was conducted at room temperature, on an anti-vibration table using a luciferin-luciferase luminometric assay. The ATP release was initially tested under non-stimulated control conditions. The amount of ATP measured in the culture medium of basolateral and apical compartments showed a tendency to be reduced in CuFi-1 cells compared to NuLi-1, but these differences were not significant (Figure 4.2.1, (A+B)). Under control conditions cells pre-treated with carbenoxolone (10 µM) a general connexin inhibitor which has the ability to inhibit panexin channles and probenecid (10 µM) a more specific pannexin-1 channel inhibitor significantly reduced the amount of ATP appearing apically in NuLi-1 epithelia (n=4, $P<0.05$ and n=3, $P<0.05$ respectively, ANOVA) but not in CuFi-1 epithelia. In the non-CF and CF cell lines, LXA_4 induced a significant release of ATP in the apical compartment without affecting the amount of ATP released into the basolateral compartment (Figure 4.2.1: (C+D)). In NuLi-1 cells, the amount of ATP in the apical compartment increased from 11.01 ± 0.48 nM (n=9) to 186.4 ± 51.04 nM (n=8, p<0.001, ANOVA) upon exposure to LXA_4 (1 nM) (Figure 4.2.1, (C)). In CuFi-1 cells, LXA_4 also stimulated a significant apical ATP release. The amount of ATP measured in the apical compartment of CuFi-1 cells increased from 11.98 ± 1.62 nM (n=9) to 279.3 ± 61.02 (n=10, $P<0.001$, ANOVA) upon exposure to LXA_4 (Figure 4.2.1 (D)). The pre-treatment with antagonists of pannexin-1 channels, carbenoxolone (10 µM), probenecid (10 µM) and 10Panx, a pannexin-1 mimetic inhibitory peptide (100 µM) for 15 min, all completely abolished the effect of LXA_4 on apical ATP release in NuLi-1 and CuFi-1 cell line (Figure 4.2.1: (C+D)). In reconstituted primary CF bronchial epithelial cells blocking the FPR2 receptor with the Boc-2 antagonist resulted in a decrease in the amount of ATP released apically. The decrease was also observed when Panx-1 channel was blocked by 10Panx (100 µM) (Figure 4.2.2). This result indicates that LXA_4 stimulates an apical ATP release through Panx1 channels. These results indicate that LXA_4 induces a release of ATP into the apical ASL via pannexin-1 channels in non-CF (NuLi-1) and CF (CuFi-1) and primary CF bronchial epithelial cells when these cells are fully differentiated.

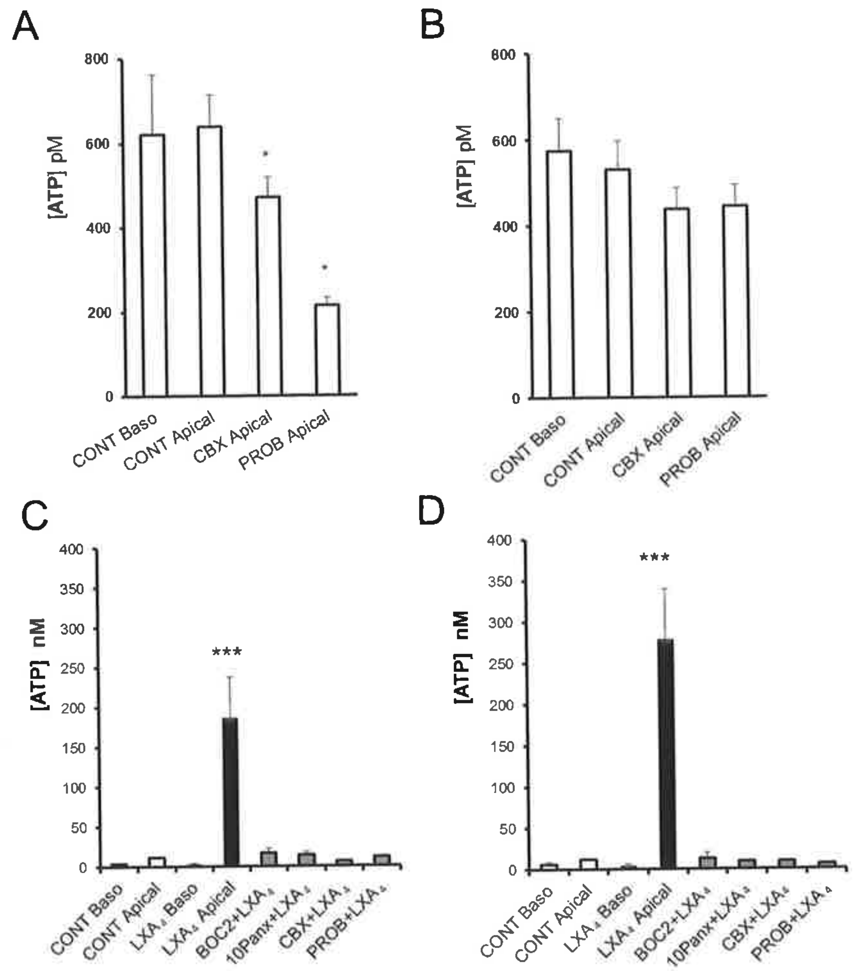


Figure 4.2.1: ATP release induced by LXA_4 is through pannexin-1 channels. (**A+B**) Basal levels of ATP measured in apical and basolateral culture compartments of NuLi-1 (A) and CuFi-1 (B) cells grown under an air-liquid interface. Effect of carbenoxolone (CBX, 10 μM) and probenecid (PROB, 10 μM) on spontaneous apical ATP release in NuLi-1 and CuFi-1 (*P* * ≤0.05, ANOVA). Stimulation of LXA_4 (1 nM) results in a significant increase in the apical ATP release by NuLi-1 and CuFi-1 cell lines (**C** and **D** respectively) (*P* *** ≤ 0.001, ANOVA). The increase of apical ATP release was inhibited in NuLi-1, CuFi-1 cells by Boc-2 (10-5 M), 10Panx (100 μM), carbenoxolone (CBX, 10 μM), probenecid (PROB,10 μM) and nocodazol (NOCO, 20 μM) (P^{***}≤ 0.001, ANOVA) (**C** and **D**).

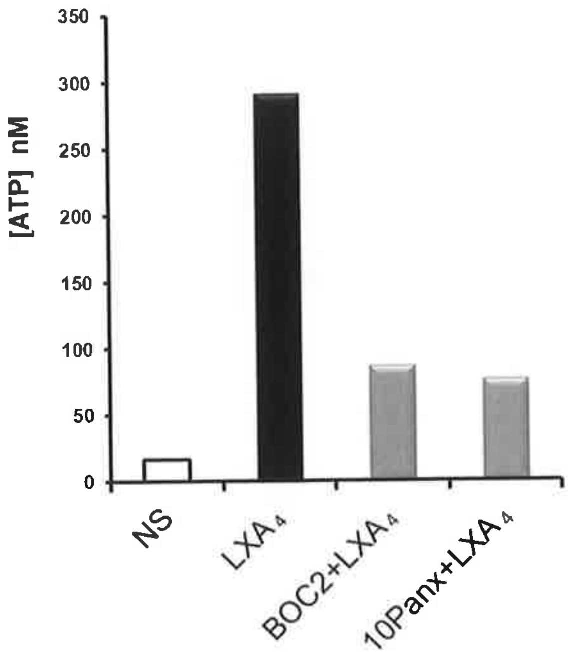


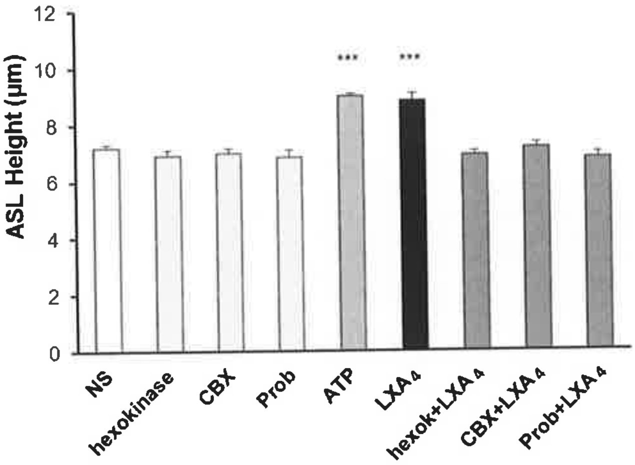
Figure 4.2.2: ATP release induced by LXA_4 is through pannexin-1 channels in CF primary epithelia. Stimulation of LXA_4 (1 nM) results in an increase in the apical ATP release by CF primary cultures compared to non-stimulated conditions (NS). The increase of ATP release induced by LXA_4 was also inhibited in CF bronchial epithelial cells when the ALX/FPR2 (Boc-2) receptor and Panx1 (10Panx) channels were blocked.

4.3.2. The role of ATP in regulating ASL height

The effect of the ATP secretion induced by LXA_4 on the ASL height was investigated using live cell confocal microscopy. The hydrolysis of external ATP using hexokinase and the inhibition of ATP release with pre-treatment of carbenoxolone (10 μM) and probenecid (10 μM) 15 min prior to stimulation with

LXA_4 did not significantly affect the ASL height in NuLi-1 (Figure 4.3, (A)) and CuFi-1 (Figure 4.3, (B)) cell line epithelia under basal conditions but did inhibit the ability of LXA_4 to stimulate an increase in ASL height. LXA_4 (1 nM) significantly increased the ASL height from 7.2 ± 0.11 µm (n=4) to 8.8 ± 0.24 µm (n=4, *P* <0.001, ANOVA) in NuLi-1 cells and from 5.7 ± 0.11 µm (n=5) to 7.5 ± 0.06 µm (n=5, *P* <0.001, ANOVA) in CuFi-1 cells. Externally added ATP (10 µM) to the apical chamber mimicked the effect of LXA_4 by increasing the ASL height to 9.1 ± 0.19 µm in NuLi-1 cells (n = 4, *P* <0.001, ANOVA) and 8.2 ± 0.05 µm in CuFi-1 cells (n = 5, *P* <0.001, ANOVA). In NuLi-1 cells, the ASL height measured after cells were pre-treated by hexokinase (10 µM), carbenoxolone (10 µM) or probenecid (10 µM) prior to LXA_4 stimulation were 6.9 ± 0.15 µm (n=4), 7.1 ± 0.16 µm (n=4) and 6.8 ± 0.19 µm (n=4), respectively. In CuFi-1 cells, the ASL height measured after exposure to hexokinase, carbenoxolone and probenecid prior to LXA_4 stimulation was 5.9 ± 0.15 µm (n=4), 5.9 ± 0.18 µm (n=4), and 5.4 ± 0.12 µm (n=4) (Figure 4.3: (A and B)). Taken together these results indicate that the apical ATP release induced by LXA_4 mediates the stimulatory effects of LXA_4 on the ASL height in non-CF and CF differentiated bronchial cell lines.

A



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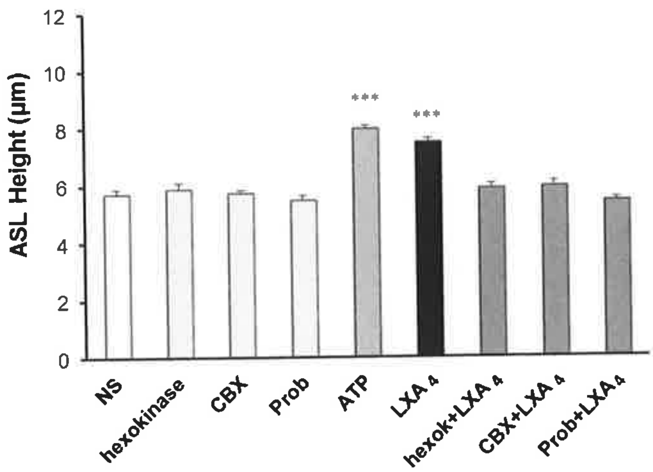


Figure 4.3: LXA_4 induced ATP release increases ASL height in CF airway epithelial cell lines. Mean height of ASL in NuLi-1 (**A**) and CuFi-1 (**B**) bronchial epithelium cell lines. LXA_4 mimicked the ASL height increase induced by ATP. The ASL height increase was abolished by hexokinase (10 µM), carbenoxolone (CBX, 10 µM) and probenecid (PROB, 10 µM) inhibitors of the panexon channels in NuLi-1 and CuFi-1 cells (*P* ***≤ 0.001, ANOVA) returning ASL height to that of non-stimulated conditions (NS).

4.3.3. Purinoreceptors are transducers for the effects of LXA_4 on ASL height in bronchial epithelium

The role of P2Y receptors in the LXA_4 mediated increase in ASL height was investigated by using a number of different inhibitors (reactive blue-2, NF340, and H89). Reactive blue 2 (10 μM) completely abolished the effect of LXA_4 on ASL height in both NuLi-1 and CuFi-1 (Figure 4.4.1, (A) and 4.4, (B) respectively) epithelia but did not significantly affect the basal ASL height in either NuLi-1 (n=12) or CuFi-1 (n=9) cells. A selective inhibitor of the P2Y11 receptor (NF340) was tested on the LXA_4 mediated effect on ASL. At concentrations of 100 nM and 1 μM, NF340 did not significantly affect the basal ASL height in either NuLi-1 (n=6 for 100 nM and n=7 for 1 uM) or CuFi-1 cells (n=4 for 100 nM and n=4 for 1 uM) . However, NF340 abolished the ASL height increase in both NuLi-1 (Figure 4.4.1, (A)) and CuFi-1 (Figure 4.4.1, (B)) cells and primary bronchial epithelial cells (Figure 4.4.2) induced by LXA_4. cAMP dependant protein kinase activity is a potent regulator of epithelial ion transport and P2Y11 potentially stimulates the cAMP signalling pathway, the effect of the PKA inhibitor, H89 was also tested on the ASL response to LXA_4. H89 partially inhibited the up-regulation of ASL height by LXA_4 suggesting a role for PKA in this effect. It has been reported that adenosine receptor stimulation might also regulate airway epithelial ASL height via a cAMP dependent signalling pathway. However, alloxazine (10 μM) used as an inhibitor of A_2b receptor, did not affect the response to LXA_4 (Figure 4.4.1, A and B). These results suggest a role for an apical P2Y11 receptor mediating the ASL height response to LXA_4. NF340 (100 nM) also eliminated the effect of LXA_4 on ASL height in CF bronchial epithelial primary culture (Figure 4.4.2).

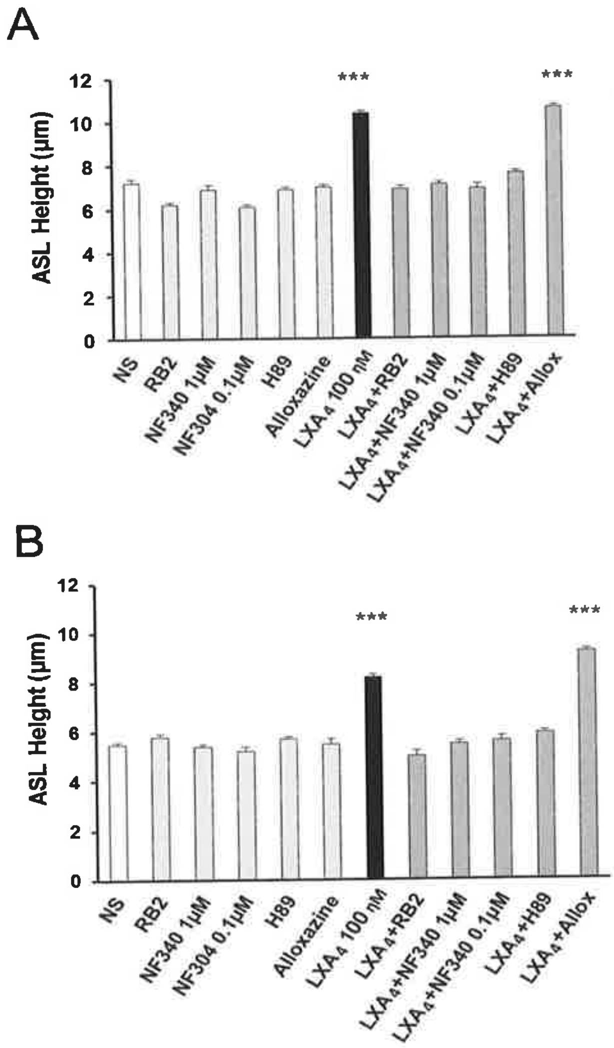


Figure 4.4.1: Purinoreceptors are responsible for the effect LXA_4 has on ASL height. LXA_4 significantly increases ASL height compared to non-stimulated conditions (NS). The increase in ASL height induced by LXA_4 is abolished in NuLi-1 (**A**) and CuFi-1 (**B**) cells when the P2Y11 receptor is inhibited by reactive blue 2 (RB2) (10µM) and NF340 (100 nM and 1 µM). The PKA inhibitor, H89 (10 µM), also eliminated the ASL height increase induced by LXA_4 in non-CF and CF cell lines. Alloxazine (10 µM) had no effect on ASL height increase measured in control conditions or upon stimulation with LXA_4 (1 nM) in NuLi-1 and CuFi-1 cells.

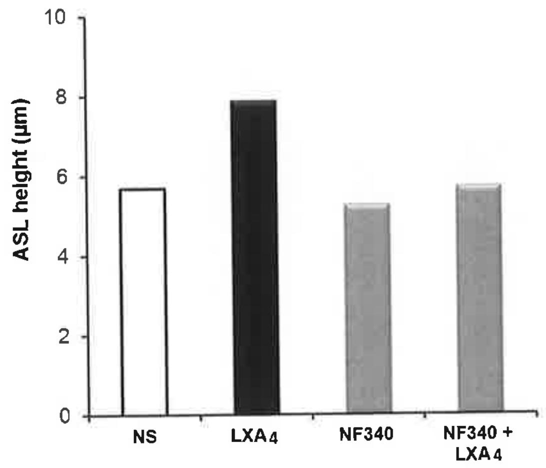


Figure 4.4.2: P2Y11 receptor is responsible for the effect LXA_4 has on ASL height in primary bronchial epithelium. The ASL height in CF bronchial epithelial primary cultures increased upon stimulation with LXA_4 compared to non-stimulated conditions (NS) in primary CF. The increase was inhibited to basal levels when the P2Y11 receptor was blocked by NF340.

4.3.4. The effect of LXA_4 on intracellular cAMP

The response of ASL to LXA_4 was antagonised by PKA inhibition, which would be coherent with an effect of LXA_4 on cAMP. Therefore the effect of LXA_4 at different concentrations on cAMP and the possible role of P2Y11 in creating this response was investigated. In NuLi-1 (Figure 4.5, (A)) and CuFi-1 (Figure 4.4, (B)) cells, LXA_4 used at 10^{-9} M (n=12 in NuLi-1 and n=12 in CuFi-1), 10^{-8} M (n=8 in Nuli-1 and n=9 in CuFi-1) and 10^{-7}M (n=10 in Nuli-1 and n=10 in CuFi-1) induced a significant increase in intracellular cAMP. The most significant cAMP rise was obtained at 100 nM LXA_4 in NuLi-1 cells, whereas the highest rise in cAMP was obtained at 1 nM LXA_4 in CuFi-1 cells. The increase in cAMP was completely abolished by inhibition of the P2Y11 receptor using NF340 (10 µM) in both NuLi-1 (n=13 for LXA_4 10^{-9} M and n=11 for LXA_4 10^{-7} M) and CuFi-1 cells (n= 13 for LXA_4 10^{-9} M and n=11 for LXA_4 10^{-7} M). These results indicate that LXA_4 generates a cAMP increase mediated by stimulation of P2Y11 receptors. (* $P < 0.05$, ANOVA).

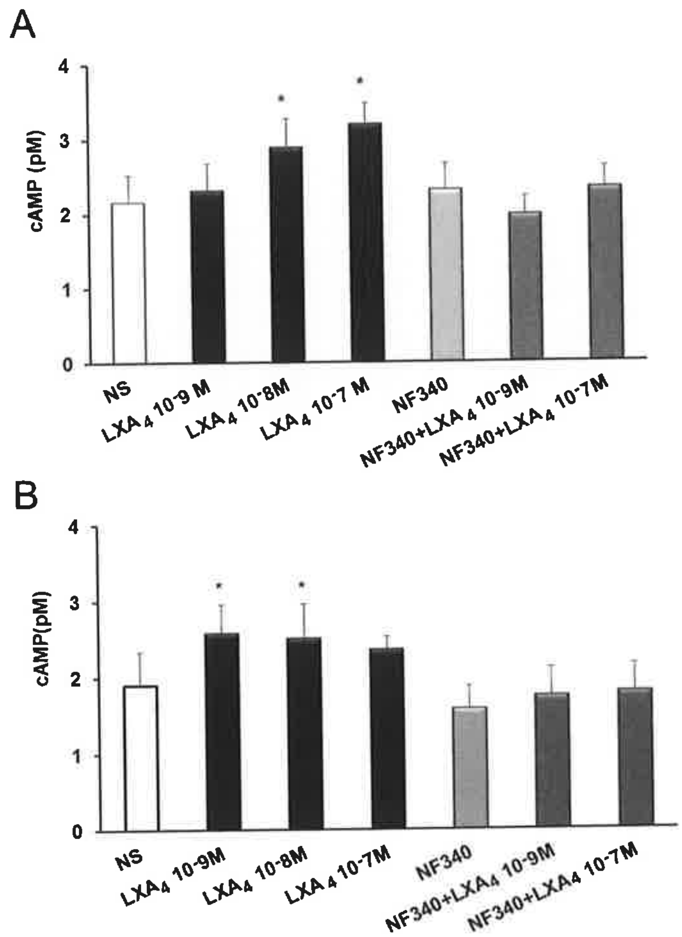


Figure 4.5: Concentration-dependent LXA_4 effects on cAMP in NuLi-1 and CuFi-1 cells. cAMP was measured using an enzyme immunoassay in both NuLi-1 (**A**) and CuFi-1 (**B**) cells following treatment with LXA_4. The effect of LXA_4 on ASL was partially inhibited by H89 a PK activity inhibitor in both cell lines. In NuLi-1 cell lines LXA_4 significantly increased cAMP most effectively at a concentration of 10^{-8} and 10^{-7} M. In CuFi-1 cells the most significant increase was at 10^{-9} and 10^{-8} M. This increase in cAMP was completely abolished by inhibition of the P2Y11 receptor with NF340 in both NuLi-1 and CuFi-1 cells. These results indicate that LXA_4 generates a cAMP increase involving the P2Y11 receptor (* $P < 0.05$, ANOVA).

4.3.5. Expression of P2Y11 in bronchial epithelial cells

Our results support a role for an apical P2Y11 receptor in transducing LXA_4 effects on ASL height. The expression and localisation of P2Y11 receptors in NuLi-1 and CuFi-1 cells upon stimulation with LXA_4 was examined. However, LXA_4 did not significantly affect the overall expression of the P2Y11 receptor measured by Western Blotting. This result was confirmed by measuring optical density with Image J (Figure 4.6, (A)). As shown on the confocal microscopy images (Figure 4.6, (B+C)) the P2Y11 receptor is expressed at the apical membrane of both non-CF (NuLi-1), CF (CuFi-1) cell lines and CF bronchial epithelium primary cultures from 2 different patients with ΔF508/508 mutation. LXA_4 (1 nM) exposure increased the apical expression of P2Y11 receptor in NuLi-1, CuFi-1 and CF bronchial epithelium primary cultures. Immunofluorescence intensity for P2Y11 was significantly increased when cells where treated with LXA_4 1 nM (*P <0.05, ***P <0.001, ANOVA) (Figure 4.5, (D)). These results indicate that LXA_4 rapidly stimulates the trafficking of the P2Y11 receptor at the membrane without affecting its total protein expression.

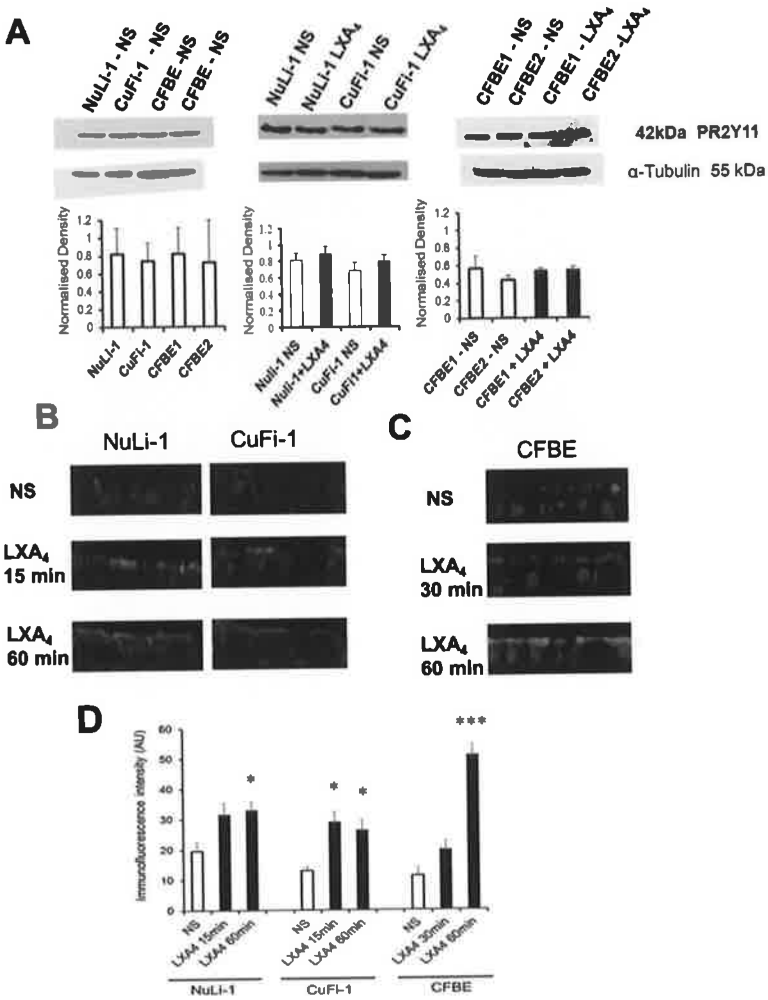


Figure 4.6: Apical expression of P2Y11 in NuLi-1 and CuFi-1 cell lines and CF bronchial epithelium primary cultures. (**A**) Western blot of typical protein expression levels of P2Y11 with LXA_4 treatment or non-stimulated (NS) conditions showed no difference in P2Y11 protein expression in NuLi-1, CuFi-1 and two different CF patients (CFBE1 and CFBE2). (**B** and **C**) Typical immunofluorescence images showing apical localisation of the P2Y11 receptor (Green Alexaflour 488) in NuLi-1 and CuFi-1 cell lines and CF bronchial epithelium (CFBE) primary cultures. P2Y11 receptor localisation upon exposure to LXA_4 for 15 min and 60 min. (**D**) Quantification of fluorescence intensity measured at the epithelial apex (n=6 for each conditions and cell type) (*$p<0.05$, ***$p<0.001$, ANOVA).

4.3.6. P2Y2 expression NuLi-1 cells CuFi-1 cells and CF bronchial epithelium primary cultures

It has been reported that P2Y2 plays a major role in ASL height maintenance. The role LXA_4 plays in receptor expression was also examined in NuLi-1, CuFi-1 and CF bronchial epithelium cells from two different CF patients both with the ΔF508/508. Expression of the P2Y2 receptor was confirmed by Western Blot analysis in NuLi-1, CuFi-1 and CF bronchial epithelium cells. Expression levels of the P2Y2 receptor did not change upon stimulation with LXA_4 (1nM) after 16hours (Figure 4.7: (A)). Rapid trafficking of the P2Y11 receptor to the apical membrane was observed after 15 min (1 nM) and increasing after 60 min upon stimulation with LXA_4 . P2Y2 receptor expression at the apical membrane was also examined by immunofluorescence as shown by confocal microscopy and confirmed by image analysis using ImageJ (Figure 4.7: (B+C)). The overall expression of the P2Y2 receptor was decreased compared to P2Y11 receptor expression (Figure 4.7: (C)). There was no change in P2Y2 receptor expression, localisation or intensity in NuLi-1, CuFi-1 and CF bronchial epithelium cells when stimulated with LXA_4. This result suggests that although P2Y2 may play a role in ASL height maintenance it is unlikely that it plays a major role in the ASL height induced by LXA_4.

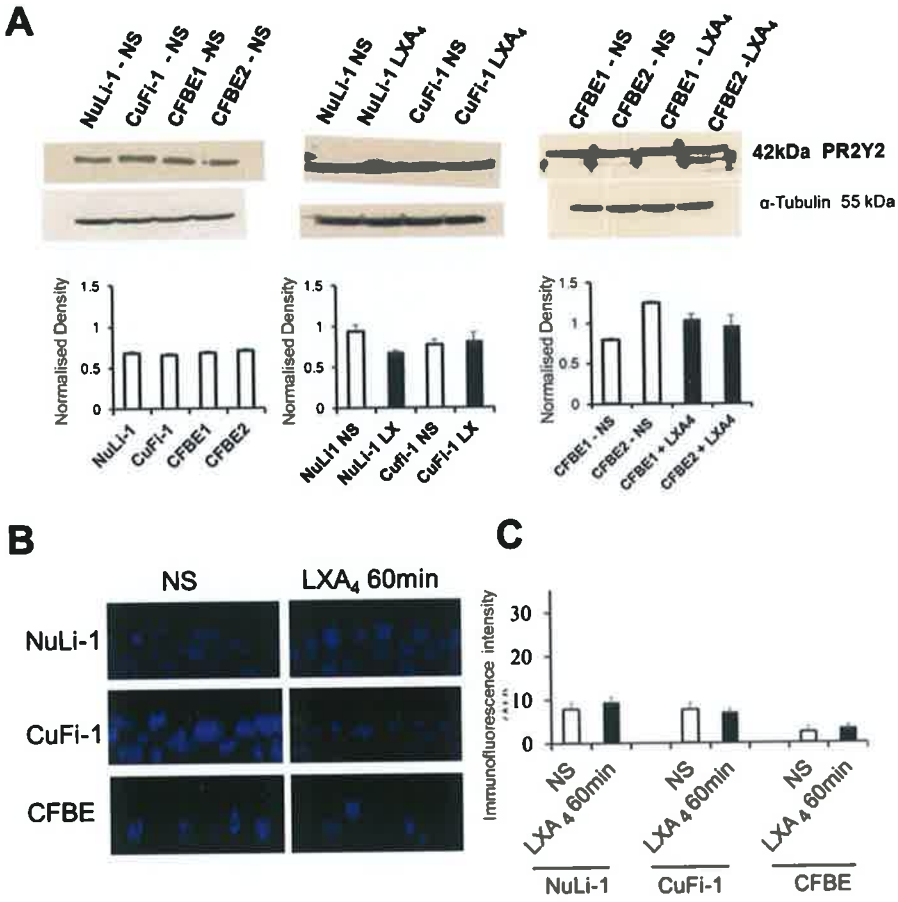


Figure 4.7: P2Y2 expression in airway epithelial cells NuLi-1 and CuFi-1cell lines and CF bronchial epithelium primary cultures. (**A**) Typical protein expression levels of P2Y2 receptor in NuLi-1, CuFi-1 and CF primary cultures from two different CF patients (CFBE1 and CFBE2) stimulated with LXA_4 or non-stimulated (NS) conditions, showed no difference in P2Y2 protein expression. (**B**) Immunofluorescence images of the P2Y2 receptor (Green) in NuLi-1 and CuFi-1 cell lines and CF bronchial epithelium primary culture with or without LXA_4 treatment brought about no changes in apical expression or intensity. (**C**) Quantification of P2Y2 staining (green fluorescence intensity) measured in control conditions (NS) or after 15 and 60 min exposure to LXA_4 (n=6 for each conditions and cell type).

4.3.7. LXA_4 increases ALX/FPR2 receptor expression in CF bronchial epithelium cells

The role LXA_4 plays on increasing the ALX/FPR2 receptor expression in primary CF bronchial epithelium cells was also investigated. Bronchial epithelium cells from 2 patients (CFBE1 and CFBE2) that have the ΔF508/508 mutation were stimulated with LXA_4 (1nM) and protein extracted for Western Blotting determination. LXA_4 increases receptor expression in CF bronchial epithelium cells compared to basal levels (n=3). The increase in ALX/FPR2 receptor expression was confirmed by measurement of optical density obtained from ImageJ (Figure 4.8: (A)).

A

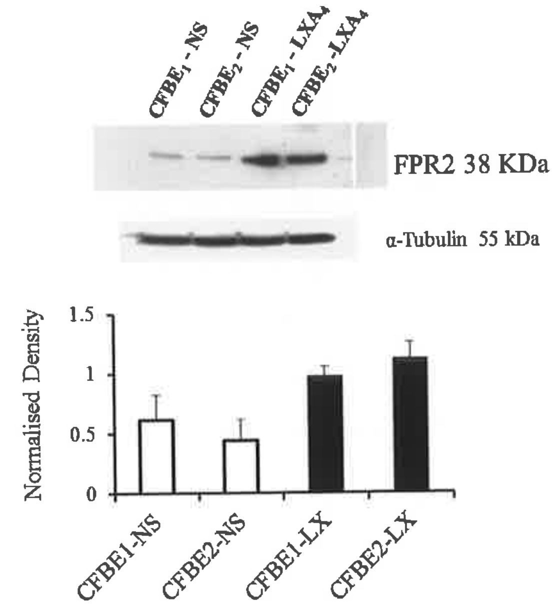


Figure 4.8: LXA_4 increases ALX/FPR2 receptor expression in CF bronchial epithelium cells. LXA_4 increased protein expression of whole cell lysates of the ALX/FPR2 receptor in primary CF bronchial epithelium from two different patients when compared to non-stimulated cells was confirmed by optical density.

4.4. Discussion

This chapter provides evidence for the cellular mechanism by which LXA_4 induces an increase in the ASL height on bronchial epithelium. It was observed that LXA_4 causes an apical ATP secretion via pannexin-1 channels which results in the stimulation of P2Y11 purinoreceptors and subsequent Ca^{2+} and cAMP signalling.

The effect of Boc-2 as an inhibitor of the ALX/FPR2 receptor, is consistent with other reports showing that LXA_4 binds to the ALX/FPR2 receptor to produce Boc-2 sensitive effects in a number of cells including airway epithelial cells (120, 135). The increase in ALX/FPR2 receptor expression in primary CF bronchial epithelial cells is the first report of our knowledge showing an increase in ALX/FPR2 receptor expression induced by LXA_4. Previous work found that knocking down ALX/FPR2 expression by 81% using SiRNA completely abolished the effect of LXA_4 on wound repair in NuLi-1 and CuFi-1 cells (141). Taken together these results suggest that ALX/FPR2 mediates the physiological responses induced by LXA_4.

In vitro studies have demonstrated that airway epithelia can maintain a constitutive release of ATP (47). In this chapter, we have shown that under basal conditions, non-CF (NuLi-1) and CF (CuFi-1) bronchial epithelial cells secrete ATP at similar concentrations into the apical and the basolateral compartments. In addition to a constitutive release, an enhanced ATP secretion from airway epithelial cells can occur under different stimuli, for example ATP release can be enhanced for example, with mechanical stress that mimics shear stress produced by tidal breathing (60). Two general mechanisms for induced-ATP secretion have been suggested, vesicular and channel-mediated release. Evidence for a secretory pathway in the release of nucleotides from non-excitatory cells came from glucose-dependent ATP release from the yeast *Saccharomyces cerevisia* that was inhibited by brefeldin A, a Golgi disrupting agent (57, 77). Goblet-like airway epithelial cells release ATP into the lumen during Ca^{2+} regulated exocytosis of mucin granules (79). However, airway ATP release also occurs from cells that do not contain secretory granules such as human erythrocytes that release ATP under low oxygen conditions or in response to shear stress (80). Connexins and pannexins, in addition to their essential role in the intercellular gap junctions also have been suggested as diffusion pathways for ATP. The release of ATP from human bronchial epithelial cells following hypotonic shock is reduced in the presence of selective inhibitors of pannexin channels or after silencing pannexin-1 (81). The large pore of pannexin-1 channel can be permeable to second messengers such as ATP, IP_3, amino acids, arachidonic acid and its metabolites (81, 82). In this study, inhibition of connexin and pannexin-1 channels reduced the amount of ATP spontaneously released on the apical surface of NuLi-1 epithelium but not in CuFi-1 epithelium. Although the amount of spontaneous ATP released by both cell types was similar, the difference in sensitivity to connexin inhibitors suggests that different mechanisms are involved in the constitutive ATP release in resting non-CF and CF cells. These differences in connexin inhibitor-responsiveness could be due to the different secretory phenotypes in the CF and non-CF differentiated cells.

In contrast, the LXA_4 induced apical ATP release was completely inhibited by the more selective pannexin-1 channel inhibitor ^{10}Panx, in non-CF (Nuli-1) and CF (CuFi-1 and CF bronchial epithelium primary culture) differentiated epithelia suggesting that Pannexin-1 channels play a major role in the ATP response to LXA_4 compared to other release mechanisms.

The ASL layer is a major component of the mucociliary system which is necessary for optimal removal of mucus, inhaled pathogens and particles. Nucleotides were among the first potentially therapeutic agonists to restore Cl^- and fluid secretion in CF airway independent of CFTR (61). LXA_4 exerts similar effects on ASL height as ATP, which is inhibited by hexokinase and ATP release blockers. The results reported here suggest that apical ATP release may be a major cellular mediator of the LXA_4 effects on ASL action. In contrast, the absence of a significant inhibitory effect on the non-stimulated basal ASL height by hexokinase, suggests that constitutive ATP secretion is not a major factor in regulating ASL homeostasis in resting cells, at least in our cellular models.

Extracellular ATP actions are mediated by cell surface P2-purinergic receptors. Members of the 3 different classes of receptors P2X, P2Y and adenosine receptors are expressed in the apical membrane of airway epithelia (57). In previous studies, our group demonstrated that LXA_4 stimulates a transient intracellular calcium signal which is consistent with an ATP-mediated effect of LXA_4 on ASL involving one or more P2 receptor sub-types, nucleotide hydrolysis, adenosine receptor stimulation and P2 receptor desensitization. However, the calcium signal induced by LXA_4 was due to calcium release from intracellular stores rather than calcium entry (126, 129). Therefore, since ATP binding to P2X receptors results in the opening of a cation-permeable pore allowing Ca^{2+} to enter into the cell, the ATP mediated effect of LXA_4 on ASL height, most likely, did not involve P2X purinoreceptors.

The results presented here strongly indicate that the ASL height increase induced by LXA_4 involves P2Y receptors since reactive blue used as a non-specific P2Y inhibitor completely inhibited the response to LXA_4. Therefore, a role for P2Y2 was indicated in the ATP mediated effect of LXA_4 on the ASL height. NF340 has been described as a highly potent inhibitor of P2Y11 (123) .The high specificity of NF340 for P2Y11, and the complete inhibition of the LXA_4 effects on ASL height of NuLi-1, CuFi-1 cell and CFBE primary culture, suggested a major role for P2Y11 compared to P2Y2. The dominant role for P2Y11 in the response to LXA_4 might be explained by a direct role of LXA_4 on P2Y11 trafficking at the plasma membrane that is observed in in non-CF and CF bronchial epithelial cell line as well as primary culture. This is the first report to show LXA_4 can induce the trafficking of P2Y11 receptors to the apical cell membrane surface of bronchial epithelium cells. In contrast, LXA_4 did not affect P2Y2 trafficking in NuLi-1, CuFi-1 and CF primary culture.

Pharmcoalogical data has shown that the P2Y11 receptor is preferentially activated by ATP and is coupled to both the phosphoinostide and the cAMP pathways (73), which is unique among the P2Y receptor family. P2Y11 receptors provide a mechanism, in addition to activation of P2Y2 or adenosine receptors, by which exogenous or endogenously secreted nucleotides can increase cellular levels of both calcium and cAMP. In airway epithelial cells, LXA_4 stimulated both an intracellular calcium signal and a cAMP increase which is consistent with activation of Gq and Gs protein pathways via ATP binding to an apical P2Y11 receptor. These results taken together with the inhibition of the LXA_4 induced ASL height increase by NF340, and by H89, a PKA inhibitor, strongly implicate the P2Y11 receptor in transducing the epithelial responses to LXA_4.

Adenosine acts as physiological ligand that stimulates purinoreceptors to induce cAMP/PKA signal transduction pathways (62, 63). Inhibition of the A_2b receptor by adenosine desaminase in non-CF respiratory epithelial cultures caused a depletion of ASL volume, suggesting that the normal steady-state release of ATP or its metabolite adenosine is critical for a functional ASL and airway clearance. It has been proposed that adenosine addition to airway epithelial surface under basal conditions activates A_2b purinoreceptors, stimulating cAMP and CFTR sufficiently to provide CFTR-dependent regulation of ENaC and Cl^- secretion to maintain ASL at an optimal height of 7 μM ASL. In CF, the adenosine A_2b receptor system and cAMP-dependent activation of PKA are functional, but the absence of CFTR protein in the apical membrane renders the Cl^- secretory and Na^- inhibitory effects of adenosine signalling ineffective (63) . LXA_4 induced an increase in ASL height which was dependent on the cAMP and PKA signalling pathway. However, the ineffectiveness of the A_2b receptor antagonist alloxazine to inhibit the effects of LXA_4 on ASL does not support a substantial role for adenosine receptors in transducing responses of LXA_4.

In conclusion, the work reported in this chapter provides evidence for an entirely novel role for LXA_4 in restoring normal airway surface liquid dynamics, correcting ion transport dysfunction (reducing Na^+ hyperabsorption and stimulating Cl^- secretion) in fully differentiated CF airway cell cultures, by stimulating an apical ATP release and activating a purinoreceptor pathway which results in an increase of intracellular Ca^{2+}. A schematic representation of the effect of LXA_4 on ATP release and P2Y activation is demonstrated in Figure 4.9.

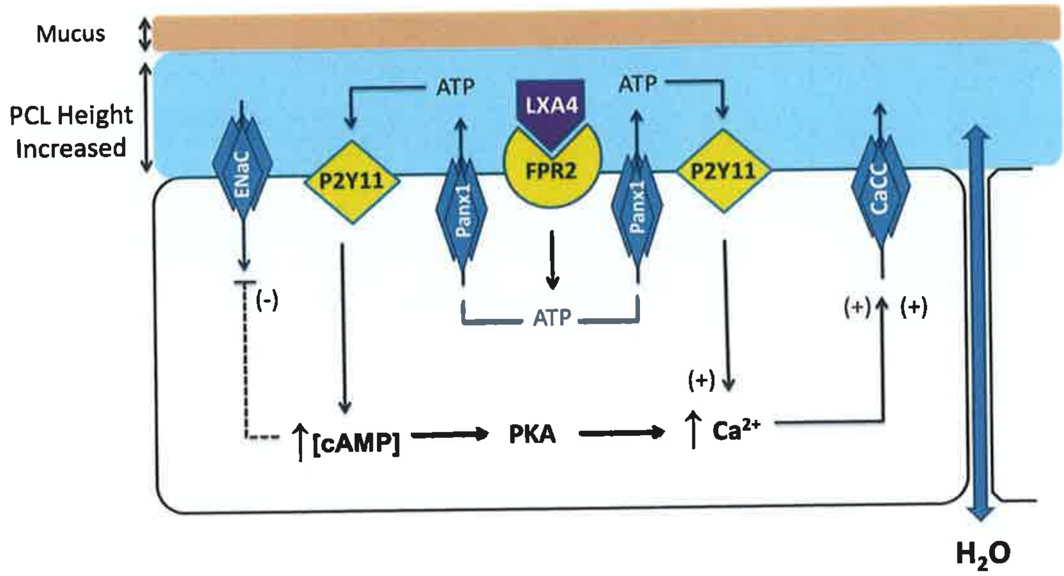


Figure 4.9: LXA_4 effect on ASL height is mediated by an apical ATP release and stimulation of P2Y11 receptors. Schematic diagram of LXA_4's effect on bronchial epithelium ion transport. LXA_4 stimulates the ALX/FPR2 receptor triggering an apical ATP release through pannexin1 (Panx1) channels. Luminal ATP stimulates P2Y11 receptors increasing intracellular cAMP and intracellular calcium (Ca^{2+}). The stimulation of a calcium activated chloride channel (CaCC) and inhibition of sodium absorption through the epithelial sodium channel (ENaC) induced by LXA_4 mediates ASL height increase in non-CF and CF bronchial epithelium cells.

Chapter V - Lipoxin A_4 delays the invasion of normal and cystic fibrosis bronchial epithelia by *Pseudomonas aeruginosa*

5.1. Introduction

As previously mentioned, levels of the eicosanoid LXA_4 are reduced in the airways of CF patients (3). Along with the role that LXA_4 has in mediating the inflammatory response, it has also been reported that LXA_4 can induce a number of physiological responses in bronchial epithelial cells for example, it has been shown to increase ASL height, increase epithelial repair (135, 141) and our laboratory has previously reported that non-CF bronchial epithelial cells respond rapidly to LXA_4 with subsequent increased tight junction formation at the apical membrane (120). We hypothesised that a decrease in LXA_4 in CF lungs could lead to increased numbers of bacteria transmigrating across the epithelial barrier.

Degradation of the bronchial epithelium within the CF lung is often a result of persistent infection and inflammation. Breakdown of the epithelial barrier function exacerbates further the infection and inflammation cycle associated with CF lung disease (Figure 5.1). *P. aeruginosa* has the ability to survive and adapt in extreme conditions, making it a formidable pathogen within the CF lung. *P. aeruginosa* infections within the lungs of CF patients are associated with the formation of biofilms on the epithelium surface. Biofilms are intrinsically resistant to antibiotics making such infections difficult to treat, with chronic infections leading to deterioration of lung function.

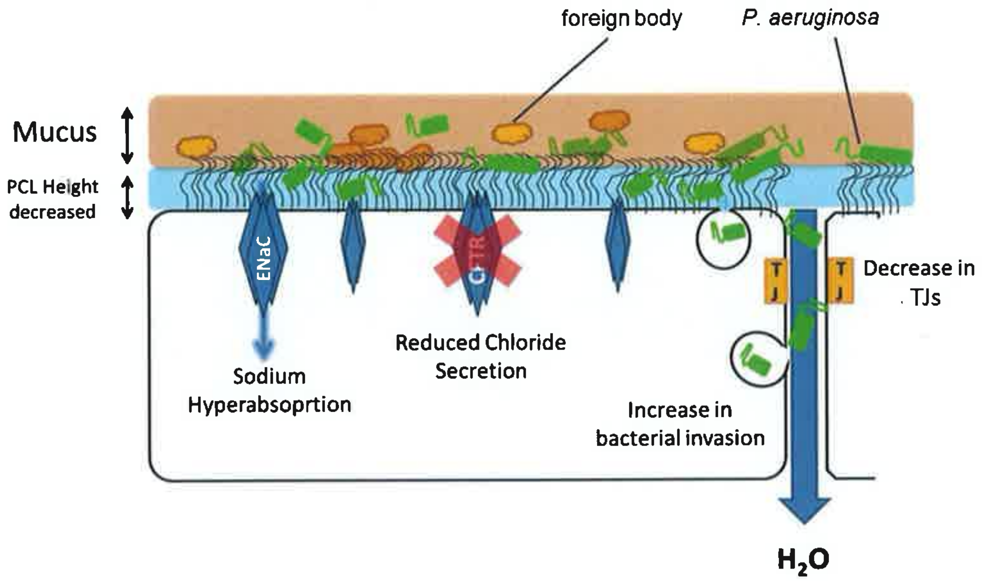


Figure 5.1: Consequences of a depleted ASL in the CF lung. The decrease in ASL height caused by the mutation of the CFTR Cl^- channel results in a defective mucociliary clearance which leads to mucus stasis, bacterial colonisation and an increased infection/inflammation cycle. This unregulated inflammation damages tight junctions (TJs). Loss of the bronchial epithelium protective barrier may lead to an increase in bacteria invading bronchial epithelial cells and transmigrating across the epithelial barrier.

By increasing ASL height and tight junction formation, LXA_4 may play an important role in protecting the bronchial epithelium. This physiological effect could potentially delay the initial attachment or invasion of bronchial epithelial cells by pathogens, such as *P. aeruginosa*. The multifunctional role of LXA_4 within the CF lung may help alleviate the decline of lung function within CF patients by strengthening the integrity of the bronchial epithelial protective layers while still modulating the inflammatory response.

5.1.2. Aim

To investigate the role of LXA_4 in delaying the invasion of non-CF and CF bronchial epithelial cells by *P. aeruginosa*. To test this hypothesis, an *in vitro* model that could mimic, in part, the pathogenesis of infection by *P. aeruginosa* of the bronchial epithelial layer was developed. Within this model, epithelial cells were treated with LXA_4 and subjected to a gentamicin exclusion assay. From this, the number of bacterial cells that invaded the epithelial layers was deduced.

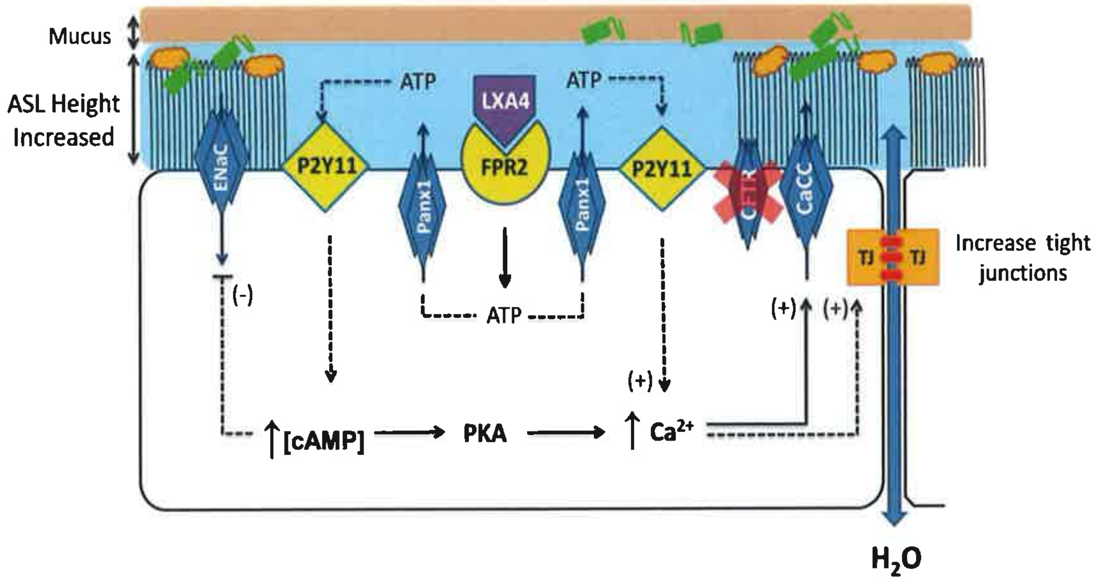


Figure 5.2: The role of LXA_4 in protecting bronchial epithelium from invasion of bacteria. The epithelial barrier integrity is protected by an increase in ASL height induced from purinoreceptor (P2Y11) stimulation by ATP activating chloride secretion through a calcium activated chloride channel (CaCC) which in turn inhibits the sodium epithelial channel (ENaC) and by an increase in tight junction (TJ) formation at the apical membrane. It is proposed that these physiological effects induced by LXA_4 may delay the invasion of bronchial epithelial cells by *P. aeruginosa*.

5.2. Specific Methodologies

5.2.1 Bacterial strains and clinical isolates

P. aeruginosa strain PAO1 was obtained from Professor Fergal O'Gara, University College Cork, Ireland

*P. aeruginosa*1 was isolated from a sputum specimen obtained from a CF patient that was sent for routine diagnostic tests at the Microbiology Laboratory, Our Lady's Children's Hospital, Crumlin, Dublin 12. Ireland. It has a non-mucoid phenotype. The isolate is fully susceptible to Amikacin, Ceftazidime, Ciprofloxacin, Colistin, Genatmicin and Tobramicin.

*P.aeruginosa*2 was also isolated from a sputum specimen taken from a chronically infected CF patient during routine diagnostic tests. Again, it has a non-mucoid phenotype and is resistant to Amkacin, Ceftazidime, Ciprofloxacin and Tobramicin as determined.

5.2.2. Gentamicin Exclusion Assay

Primary CF bronchial epithelium, NuLi-1 or CuFi-1 cells were grown to a fully differentiated, polarised epithelium on transwell supports under an air-liquid interface. Stimulated cells were treated with LXA_4 (1 nM) for either 18 h or 24 h. PAO1, *P. aeruginosa*1 and *P. aeruginosa*2 were grown in LB broth overnight at 37^0C shaking. The bacterial solution was diluted 1 in 20 in 50/50 bronchial epithelium medium without antibiotics and grown to log phase ~OD_{600} 0.6. Upon reaching log phase bacteria were equalised to an OD_{600} 0.2 in 1 ml of fresh 50/50 bronchial media warmed to 37^0C. Then, 25 µl of the bacterial suspension was added apically for 2 h on primary CF bronchial epithelial cells. The inoculating time was varied to include 1 h - 4 h during NuLi-1 and CuFi-1 invasion assays. Following this, the epithelial cells were washed and treated apically with gentamicin for 1 h to kill any extracellular bacteria. A sample of basolateral media was taken at this time, serially diluted and cultured on LB agar and incubated at 37^0C overnight. Following the gentamicin treatment, epithelial cells were washed twice apically with PBS and treated with Triton-X 100 (0.4% v/v) to lyse epithelial cells and release any internalised bacteria for 15 min. The lysate was serially diluted 1 in 10 and plated on LB agar and incubated at 37^0C overnight. CFU was recorded (accurate range ≥30≤300) and the number of CFU/ml was determined. An outline of a gentamicin exclusion assay is illustrated in Figure 5.3.

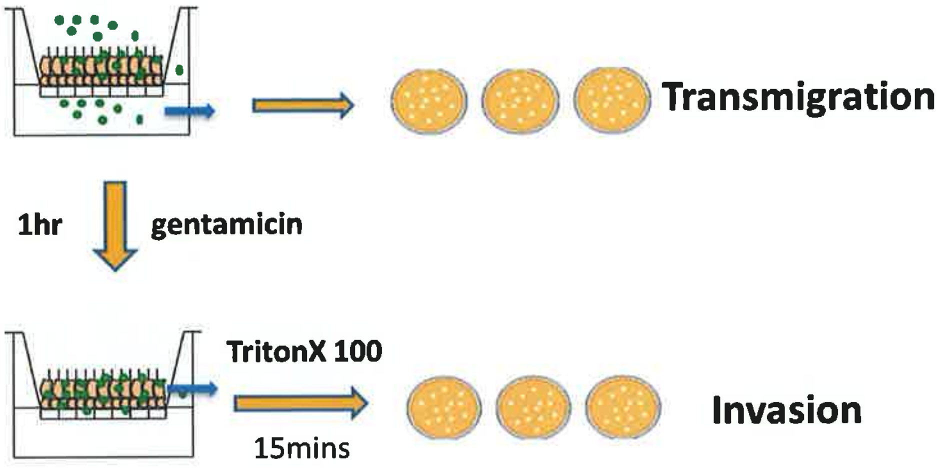


Figure 5.3: Schematic representation of a gentamicin exclusion assay. Bacteria (green) were added apically to the bronchial epithelium model. Following the required time of infection bacteria that transmigrated to the basolateral compartment were cultured on LB agar and CFU/ml determined. The epithelium was treated with gentamicin for 1 h to kill any extracellular bacteria. Bacteria that had invaded epithelial cells were retrieved by lysing the cell with Triton-X100, cultured on LB agar and CFU/ml attained.

5.2.3. Data analysis

NuLi-1, CuFi-1 experiments were repeated on three different cell passages under the same conditions and n refer to the number of inserts used. Primary bronchial cells were obtained from children under 6 years of age (patient numbers HCFBE14, HCFBE16 and HCFBE20; see appendix) Data is presented as mean ± S.E.M. of n experiments. Statistical significance was obtained using a Student T test or the one way ANOVA test. Comparisons within groups were made using the post hoc test, Newman-Keuls. In all tests a *P* value of ≤ 0.05 was deemed significant. All statistical analyses were performed using GraphPad Prism.

5.2.4. Methods - Determining the effective dose of Gentamicin for *P. aeruginosa* exclusion assays

It was necessary to determine the most effective dose of Gentamicin that would prevent the growth of *P. aeruginosa* cells that had not invaded epithelial cells during invasion assays.

The minimal bactericidal concentration (MBC) was determined as follows. *P. aeruginosa*1 and *P. aeruginosa*2 were grown overnight at 37^0C in LB broth (Sigma) with shaking. Following this, a 1:20 dilution of the overnight culture was prepared in sterile broth and grown to an OD_{600} 0.2 using a spectrometer (Jenway 6305 spectophotemeter). In separate tubes, various concentrations of gentamicin (10 mg/ml) were prepared in sterile LB broth. The final concentrations of gentamicin used were 1000, 400, 200, 100, 50, 12.5, and 1.5 µg/ml. 25µl of the bacterial suspension (OD_{600} 0.2) was added to the gentamicin solutions. Each tube was incubated overnight at 37^0C with shaking. The MBC was determined by creating a 1:10 dilution series of each test containing gentamicin. From each dilution 100µl was inoculated onto LB agar using a spreader. Plates were incubated overnight at 37^oC and the number of bacterial colonies were counted (accurate range $\geq 30 \leq 300$) and the CFU/ml determined. The MBC is defined as the minimal concentration of antibiotic required to kill the bacteria and can be seen in Figure 5.4. From these experiments 200 μg/ml of gentamicin was deemed to be most effective and was therefore used for exclusion assays.

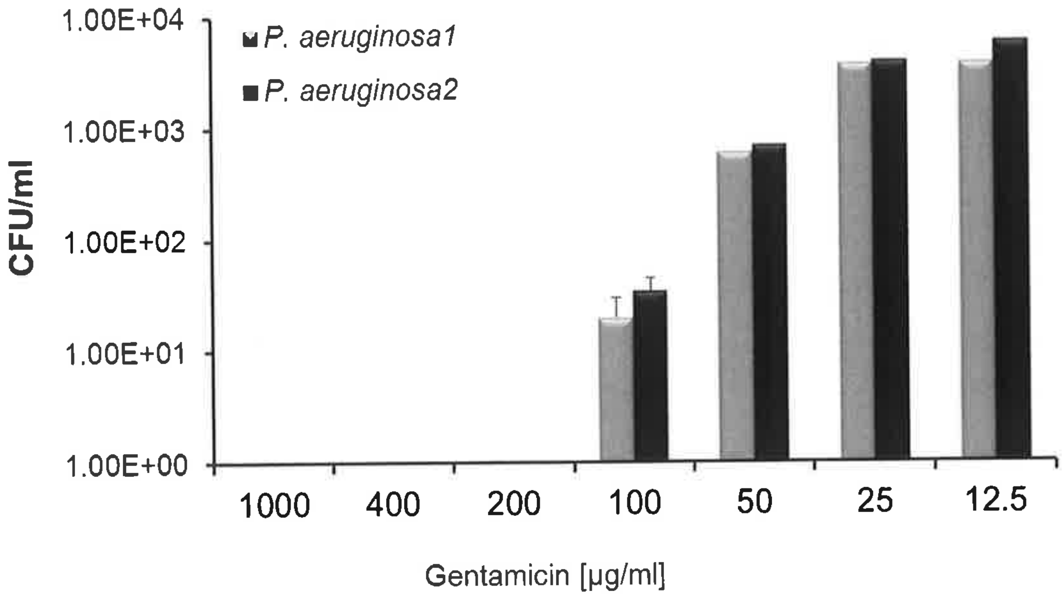
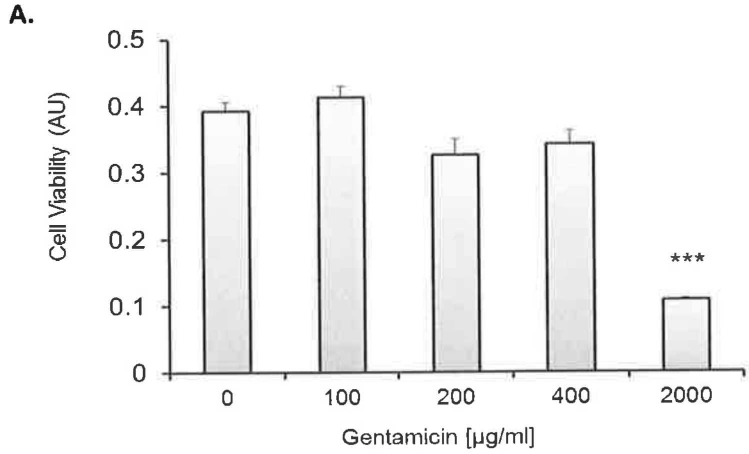


Figure 5.4: Determining the minimum bactericidal concentration of gentamicin for *P. aeruginosa*1 and *P. aeruginosa*2. The growth of *P. aeruginosa*1 and *P. aeruginosa*2 in the presence of different concentrations of the antibiotic gentamicin was determined. No growth of *P. aeruginosa*1 and *P. aeruginosa*2 was observed at concentrations of gentamicin at 1000 μg/ml, 400 μg/ml and 200 μg/ml. Growth of *P. aeruginosa*1 and *P. aeruginosa*2 was observed at 100ug/ml of gentamicin (n=3).

5.2.5. Methods - Cell Viability Assay

A cell viability assay was performed to determine if the amount of gentamicin used in the exclusion assay had any cytotoxic effects on cultured NuLi-1 and CuFi-1 cells. NuLi-1 and CuFi-1 cells were grown for two days on a 48 well tissue culture plate, as described before, and then exposed to differing concentrations of gentamicin (0 µg-2000 µg/ml) for 24 h at 37^{0}C. Cells were then exposed to MTT, or 3,-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltet razolium bromide, prepared in culture media for 4 h to allow for the formation of purple formazan crystals. The media and MTT mix was removed DMSO added to dissolve the crystals. The intensity of the coloured solution was measured at 570 nm using a colorimeter (Biotek,UK). Absorbance levels were recorded and cell viability was determined from negative and positive controls.

NuLi-1 and CuFi-1 cell viability was investigated by treating epithelial cells with 0, 100, 200, 400 ug/ml and 2 mg/ml of gentamicin. NuLi-1 cells treated with gentamicin resulted in no significant difference in cell viability when cells were treated with 0 µg/ml (0.39 AU ± 0.01, n=3), 100 µg/ml (0.41 AU ± 0.01, n=6,), 200 µg/ml (0.32 AU ± 0.02, n=6,) and 400 µg/ml (0.34 AU ±, n=6,) of gentamicin. The positive control of gentamicin (2mg/ml) decreased cell viability (0.10 AU ± 0.002, n=3, $P\ ^{***}\leq 0.001$, ANOVA) (Figure 5.5: (A)). An MTT assay was also performed on the CF epithelial cell line (CuFi-1) treated with 0 µg/ml (0.14 AU ± 0.008, n=3,), 100 µg/ml (0.15 AU ± 0.007, n=6,), 200 µg/ml (0.14 AU ± 0.009, n=6,) and 400 µg/ml (0.11 AU ± 0.008, n=6,) of gentamicin, with no significant difference in cell viability recorded. However a significant decrease was observed with the positive control of 2 mg/ml (0.07 AU ± 0.005, n=3, ANOVA) of gentamicin from 400 µg/ml (*P* **≤ 0.01) and from 0.0-200µg /ml (*P* ***≤ 0.001) (Figure 5.5: (B)). These findings indicate that the concentration of gentamicin used in the gentamicin exclusion assays will not affect bronchial airway epithelial cell viability.



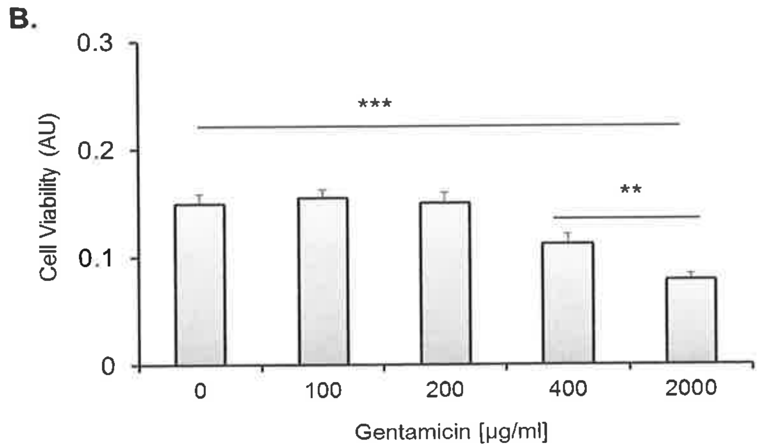


Figure 5.5: Gentamicin (200 µg/ml) does not affect epithelial cell viability of NuLi-1 and CuFi-1 cells. Cell viability of NuLi-1 and CuFi-1 cells post treatment with gentamicin (0-2000 µg/ml) was assessed using a MTT viability assay. (A) NuLi-1 cells showed no significant difference of cell viability when treated with gentamicin (0-400 µg/ml) (n=3). However a significant decrease in cell viability was observed in NuLi-1 cells at the higher concentration of 2 mg/ml (*P* ***≤ 0.001, n=3, ANOVA). (B) There was no difference in CuFi-1 cells viability when treated with different concentrations of gentamicin (0 µg/ml-400 µg/ml). At higher concentration of gentamicin 2 mg/ml viability was significantly inhibited compared to 0 µg/ml-200 µg/ml (*P* ***≤ 0.001, n=3, ANOVA) and 400 µg/ml (*P* **≤ 0.01, n=3, ANOVA) of gentamicin.

5.3. Results

5.3.1. LXA_4 delays the invasion of CF bronchial epithelia by the pathogen *P. aeruginosa*.

It was hypothesised that LXA_4 (1 nM) could delay the invasion and transmigration of bacteria across the epithelial barrier. To test this hypothesis a gentamicin exclusion assay was performed on fully differentiated and well polarised epithelia on transwell supports. Primary cultures of CF bronchial epithelial cells were inoculated with the laboratory strain PAO1 for 2 h. On completion of the gentamicin exclusion assay, a significant decrease in the amount of PAO1 invading epithelial cells 18 h post stimulation (2.3E+03 ± 1018.03, n=3) and 24 h (600 ± 126.0, n=3) post stimulation with LXA_4 compared to non-stimulated epithelial cells was observed (1.7E+04 ± 7269.07, n=3) (*P* ** ≤0.01, ANOVA) (Figure 5.6: (A)). PAO1 that transmigrated to the basolateral compartment was also recorded. In non LXA_4 stimulated CF bronchial epithelial cells, a significant increase in the number of bacteria that had transmigrated through the epithelia barrier to the basolateral compartment was also observed (461.11 ± 86.4, n=2) compared to CF bronchial epithelial cells treated with LXA_4 (1 nM) for 18 h (200 ± 45.5, n=2) and 24 h (133.33 ± 17.2) (*P* *** ≤0.001, ANOVA) (Figure 5.6: B).

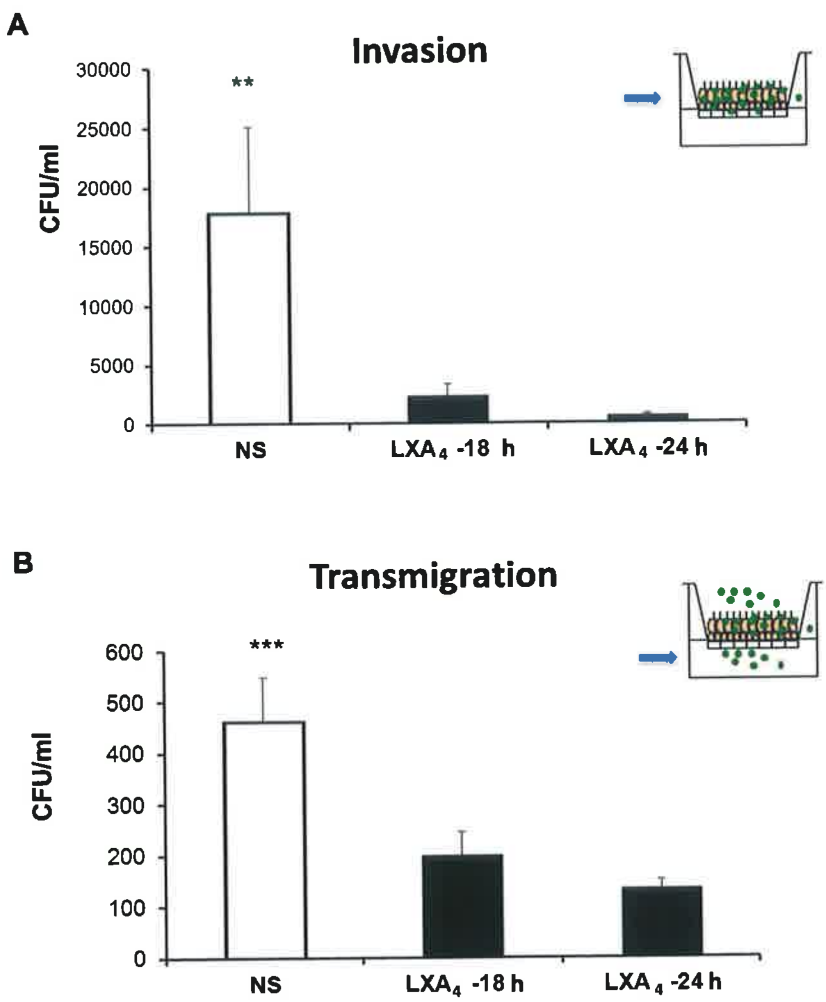


Figure 5.6: LXA_4 delayed the invasion of CF bronchial epithelial cells by the pathogen *P. aeruginosa*. Gentamicin exclusion assay was performed on primary CF bronchial epithelium cells with PAO1. (**A**) A decrease in the number of PAO1 invading CF bronchial epithelial cells was observed when epithelial cells where treated with LXA_4 (1 nM) for 18 h and 24 h (*P* **≤ 0.01, n=3, ANOVA) compared to non-stimulated CF bronchial epithelial cells. (**B**) The number of PAO1 cells that transmigrated to the basolateral compartment after 2 h infection also decreased when epithelial cells where treated with LXA_4 for 18 h and 24 h prior to inoculation (*P* ***≤ 0.001, n=3, ANOVA).

5.3.2. Inhibition of the LXA_4 receptor abolishes the delay of invasion by PAO1 in CF Bronchial Epithelial cells

The role of the ALX/FPR2 receptor (LXA_4 receptor), which is apically expressed in bronchial epithelium, was examined by pre-treating the epithelial cells for 15 min using the receptor antagonist Boc-2. A decrease in the number of PAO1 cells invading primary CF bronchial epithelial cells was observed when the latter were treated with LXA_4 (1 nM) for 24 h prior to inoculation (1.9E+03 ± 174, n=3) compared to non-stimulated CF bronchial epithelium cells (7.6E+03 ± 554.8, n=3) (*P* ** ≤0.01, ANOVA). The decrease in invading bacteria, as a result of LXA_4, was completely abolished upon treatment with Boc-2 (6.9E+03 ± 228.1, n=3) (*P* ** ≤0.01, ANOVA) (Figure 5.7).

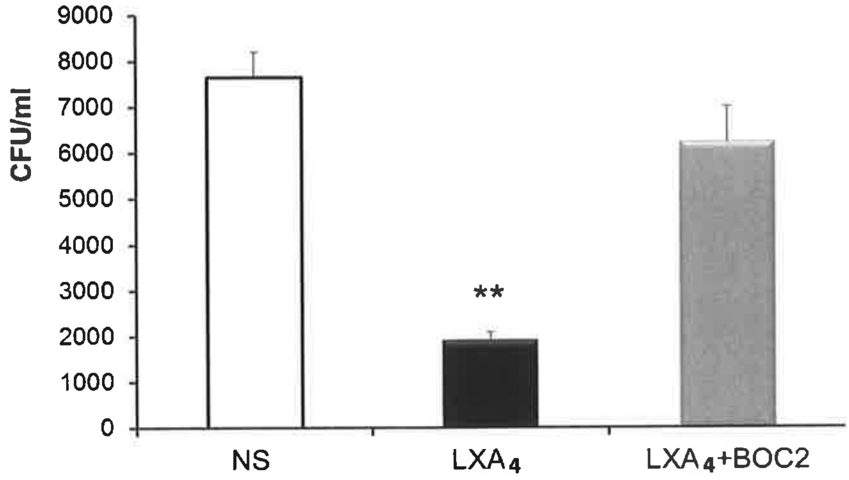


Figure 5.7: Inhibition of the ALX/FPR2 receptor abolished the delay of invasion by PAO1 in CF bronchial epithelia. CF bronchial epithelia pre-treated with LXA_4 24 h prior to inoculation with PAO1 resulted in a decrease in the number bacteria invading CF bronchial epithelia compared to non-stimulated (NS) cells 2 h post inoculation. The ability of PAO1 cells to invade CF bronchial cells 2 h post inoculation was restored when epithelial cells were pre-treated with the ALX/FPR2 receptor antagonist Boc-2 (10 μM) (*P* **≤ 0.01, n=3, ANOVA).

5.3.3. LXA_4 delays the invasion of NuLi-1 cells by clinical isolate *P.aeruginosa*1

The delay of invasion by *P. aeruginosa* was further evaluated using the clinical isolate *P. aeruginosa*1 by including the additional time points of 1, 3, and 4 h post-inoculation into the experimental procedure. NuLi-1 cells (non-CF) where treated with LXA_4 (1 nM) or left untreated 24 h prior to inoculation with *P. aeruginosa*1 cells. The number of CFU/ml calculated represented the amount of bacteria invading or transmigrating to the basolateral compartment. A decrease in the amount of invading *P. aeruginosa*1 is observed when NuLi-1cells where pre-treated with LXA_4 (1 nM) at 1 h post-inoculation (8.6E+03 ± 1.8E+03, n=3) compared to non-stimulated NuLi-1 cells (1.1E+04 ± 1.9E+03, n=3). A significant decrease in invading bacteria is also observed when NuLi-1 cells were treated with LXA_4 (1nM) at 2 h post-inoculation (4.0E+04 ± 7.6E+03, n=3) (*P* ** ≤0.01, ANOVA), 3h post-inoculation (5.2E+04 ± 8.7E+03, n=3) (*P* *** ≤0.001, ANOVA) and 4h post-inoculation (9.2E+03 ± 1.2E+03, n=3) (*P* *** ≤0.001, ANOVA) compared to non-stimulated cells at 2 h post-inoculation (1.0E+05 ± 1.0E+04, n=3), 3 h post-inoculation (5.3E+04 ± 1.4E+03, n=3) and 4 h post-inoculation (9.2E+04 ± 1.8E+03, n=3) (Figure 5.8: (A)). A decrease in *P. aeruginosa*1 transmigrating to the basolateral compartment can be seen when NuLi-1 cells were treated with LXA_4 (1 nM) at 1-4 h post- inoculation (5.4E+03 ± 959.5, n=3) compared to non-stimulated cells. (Figure 5.8: B). Taken together these results suggest LXA_4 may play a role in protecting epithelial cells from invasion by *P. aeruginosa*.

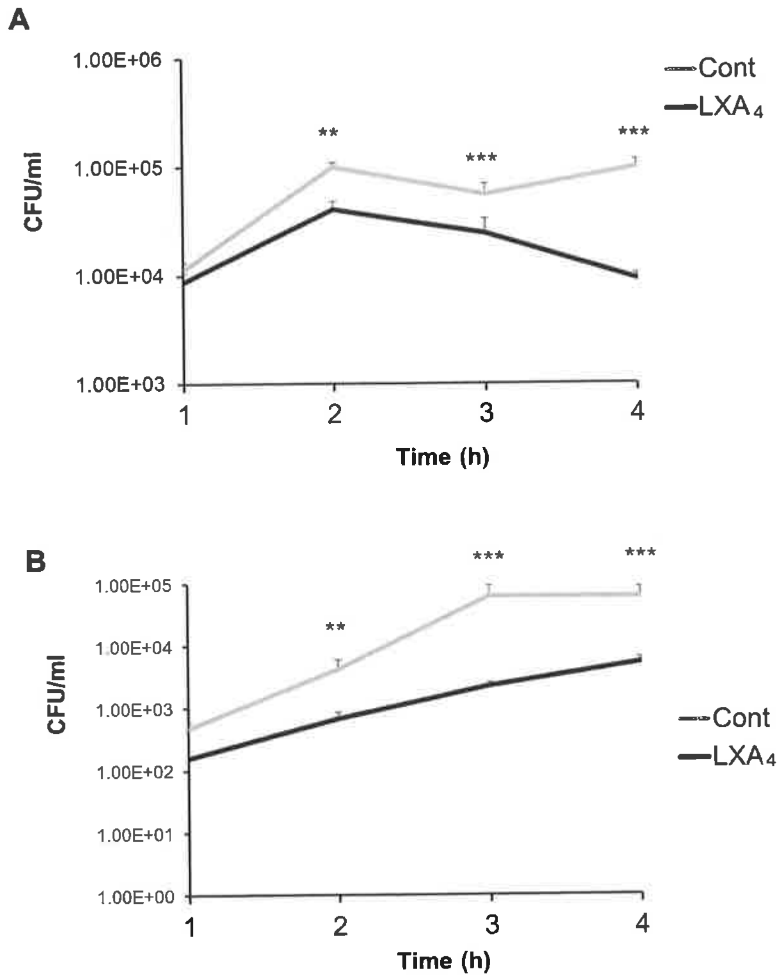


Figure 5.8: LXA_4 delayed the invasion of NuLi-1 cells by *P. aeruginosa*1.

NuLi-1 cells were treated with or without LXA_4 (1 nM) 24h prior to inoculation with *P.aeruginosa*1 cells. (**A**) Illustrates a delay in the number of *P. aeruginosa*1 invading epithelial cells at 1 h post-inoculation (n=3), 2 h post-inoculation (n=3), 3 h post inoculation (n=3) and 4 h post- inoculation (n=3) compared to controls (Cont). (**B**) Shows the number of *P. aeruginosa*1 cells that transmigrated to the basolateral compartment also decreased when NuLi-1 cells where treated with LXA_4 (1nM) 24 h prior to inoculation. The delay in transmigrating was sustained for 1 - 4 h (n=3) post infection. (*P* **≤ 0.01, *P* ***≤ 0.001, T test)

5.3.4. LXA_4 delays the Invasion of CuFi-1 cells by clinical isolate *P. aeruginosa*1.

CuFi-1 cells were also subjected to infection with *P. aeruginosa*1 with or without treatment of LXA_4 (1 nM) prior to inoculation. As before, the number of CFU/ml were calculated upon completion of a gentamicin exclusion assay. In CuFi-1 cells that were infected with *P. aeruginosa*1 a significant increase in the amount of bacteria invading epithelial cells is seen at 1 h post-inoculation (7.4E+04 ± 2.0E+04, n=3) (*P* *** ≤0.001), 2 h post-inoculation (81.5E+05 ± 1.3E+04, n=3) (*P* *** ≤0.001, ANOVA), 3 h post-inoculation (1.6E+05 ± 3.1E+04, n=3) (*P* ** ≤0.01, ANOVA) and 4-h post inoculation (9.7E+04 ± 1.6E+04, n=3) (*P* *** ≤0.001, ANOVA) compared to cells treated with LXA_4 (1 nM) at 1h post-inoculation.1E+04 ± 1.3E+03, n=3), 2 h post-inoculation (8.4E+04 ± 1.3E+04, n=3), 3 h post-inoculation (1.2E+05 ± 9.9E+03, n=3) and 4-h post inoculation (1.4E+04 ± 1.8E+03, n=3) (Figure 5.9, (A)).

The number of *P. aeruginosa*1 cells that transmigrated to the basolateral compartment, through the epithelial barrier layer, was also investigated. In CuFi-1 cells that were treated with LXA_4 (1nM) a decrease was seen in the amount of *P. aeruginosa*1 transmigrating after 1-4 h when compared to bacteria transmigrating across an un-stimulated epithelial barrier. (Figure 5.9, (B)).

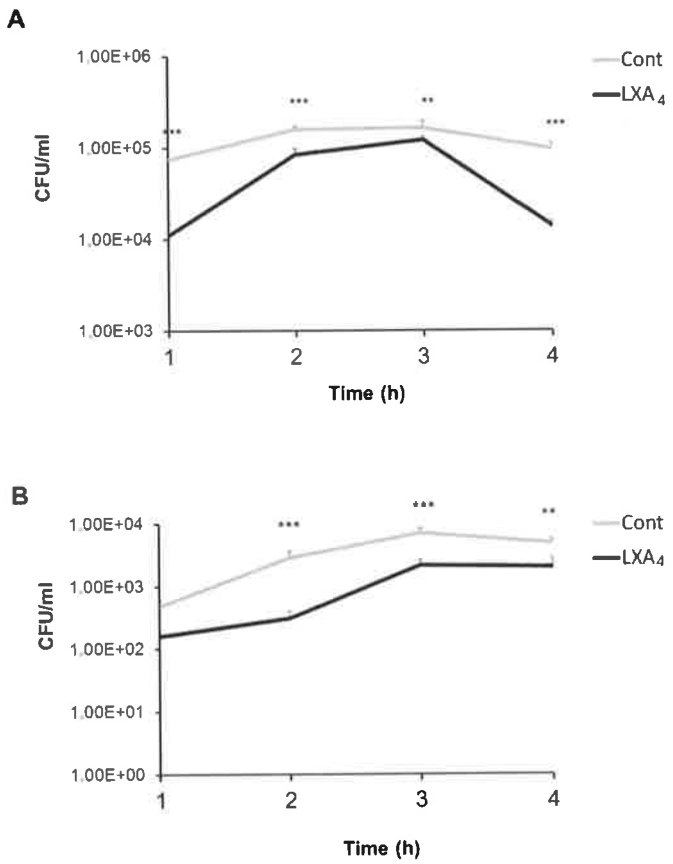


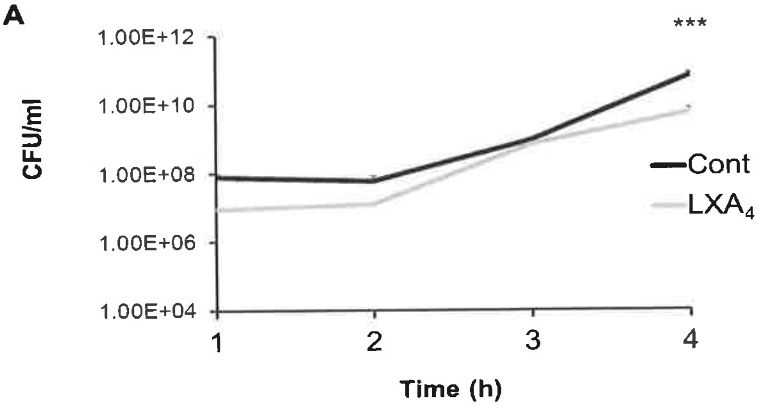
Figure 5.9: LXA_4 delays the Invasion of CuFi-1 cells *P. aeruginosa*1. The CF bronchial epithelium cell line (CuFi-1) where treated with or without LXA_4 (1nM) 24h prior to inoculation. (**A**) Illustrates delay in the number of *P. aeruginosa*1 cells invading epithelial cells at 1 h post inoculation (n=3), 2 h post inoculation (n=3), 3 h post inoculation (n=3) and 4 h post inoculation (n=3) when pre-treated with LXA_4 compared to controls (Cont). (**B**) Demonstrates that the number of *P. aeruginosa*1 cells that have transmigrated to the basolateral compartment has also decreased when CuFi-1 cells where treated with LXA_4 (1nM) 24 h prior to inoculation. The delay in transmigrating was sustained for 1-4 h (n=3) post-inoculation compared to control conditions. (*P* **≤ 0.01, *P* ***≤ 0.001, T test)

5.3.5. The treatment of NuLi-1 and CuFi-1 cells with LXA_4 delays invasion of clinical isolate *P. aeruginosa*2

The ability of a second clinical isolate, namely *P. aeruginosa*2 to invade NuLi-1 and CuFi-1 cells treated with or without LXA_4 (1nM) was also investigated over 1- 4 h. A gentamicin exclusion assay was performed and the number of CFU/ml was calculated to determine that number of bacteria invading NuLi-1 and CuFi-1 cells. The number of *P. aeruginosa*2 cells that had invaded NuLi-1 cells was increased at basal levels compared to *P. aeruginosa*1. However, similarly to *P. aeruginosa*1, there was a decrease in the amount of bacteria that had invaded NuLi-1 cells when treated with LXA_4 (1 nM) prior to inoculation at 1 h post-inoculation (9.1E+06 ± 1.0E+06, n=3), 2 h post-inoculation (1.2E+07 ± 1.2E+06, n=3) and 3 h post-inoculation (7.0E+08 ± 6.3E+07, n=3) compared to non-stimulated NuLi-1 cells at 1 h post-inoculation (7.8E+08 ± 2.4E+07, n=3), 2 h post-inoculation (6.0E+07 ± 2.27, n=3) and 3h post-inoculation (9.0E+09 ± 1.0E+08, n=3). At 4 h post-inoculation, in NuLi-1 cells treated with LXA_4 (1nM) a significant decrease was seen in the number of *P. aeruginosa*2 cells invading the epithelial cells (5.8E+09 ± 1.1E+09, n=3) (P *** ≤0.001, ANOVA) when compared to non-stimulated NuLi-1 cells (6.8E+10 ± 1.4E+10, n=3) (Figure 5.10, (A)).

Invasion of the CF bronchial epithelium cell line – CuFi-1, was also investigated with *P. aeruginosa*2. Again, there is an increase in the amount of bacteria that had invaded the CuFi-1 cells similar to NuLi-1 cells. Epithelial cells treated with LXA_4 (1 nM) results in a decrease in the amount of bacteria that invaded CuFi-1 cells 1-3 h post inoculation. At 4 h post-inoculation with *P. aeruginosa*2 cells, the number of bacteria that had invaded the CuFi-1 cells, which had been pre-treated with LXA_4 (1 nM), had significantly decreased (1.2E+10 ± 4.1E+08, n=3) ($P^{***} \leq 0.001$) compared to non-stimulated CuFi-1 cells at the same post inoculation time point (1.3E+11 ± 2.0E+10, n=3) (Figure 5.8: 10).

A



B

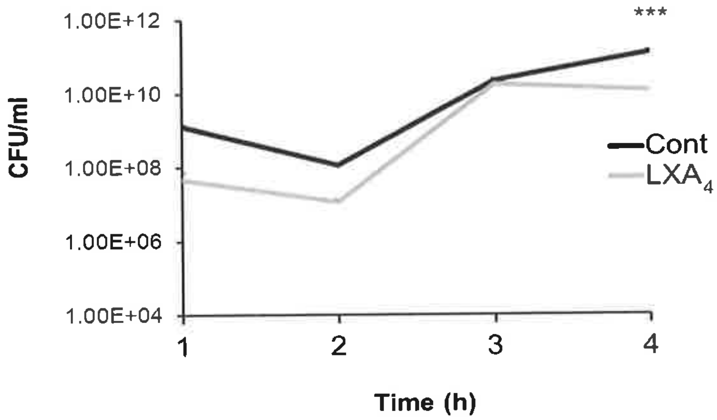


Figure 5.10: Treatment of NuLi-1 and CuFi-1 cells with LXA_4 delayed the invasion by *P. aeruginosa*2. (**A**) Treatment of NuLi-1 cells treated with LXA_4 (1nM) for 24 h, prior to inoculation resulted in a delay in the number of *P. aeruginosa*2 invading epithelial cells at 1 h – 3 h post-inoculation (n=3) with a significant decrease at 4 h post-inoculation (*P* ***≤ 0.001, ANOVA) compared to non-stimulated conditions (Cont). (**B**) The number of *P. aeruginosa*2 invading CuFi-1 cells also decreased when cells where treated with LXA_4 (1nM) for 24 h prior to inoculation. The delay in invasion was sustained for 1-3 h (n=3) post-inoculation with a significant decrease being seen at 4 h (*P* ***≤ 0.001, ANOVA) against control conditions.

5.4. Discussion

The airways of CF patients are repeatedly exposed to inflammatory insult, which in turn can lead to damage and remodelling of the airways. As a result, CF lungs are especially vulnerable to *P. aeruginosa* colonisation and infection (142, 143). *P. aeruginosa* can be described as an opportunistic pathogen that does not usually infect normal tissue in healthy individuals. Instead, this pathogen preferentially infects injured or repairing tissue such as that found in airway epithelium of CF patients. The factors that contribute to the pathogenesis of *P. aeruginosa* infection in CF is not yet fully understood. Perhaps, the deregulation of the infected airways, caused by the reduction in the availability of the lipid mediator LXA_4, is also a major contributing factor. For this reason, the physiological response of bronchial epithelia in the presence of *P. aeruginosa* and LXA_4 was examined.

Bacterial laboratory strains, such as PAO1, are reliable but constant passage can leave them with attenuated virulence. In comparison clinical isolates can be highly variable and can often display great differences even between clinical isolates of the same species due to the clonal variability that may be present in the circulating population. Although they can be difficult to work with, there are several benefits for using clinical isolates *in vitro*. For example, clinical isolates are more clinically relevant and a truer representation of what could be happening *in vivo* during infection as they are less likely to be attenuated due to constant passage in the laboratory.

Bacteria were grown in 50/50 bronchial epithelium media rather that artificial liquid broth before inoculation for a number of reasons. Firstly, it is more physiologically relevant for both the bacteria and epithelial cells in our infection model; secondly the bacteria experience a slower growth in 50/50 bronchial epithelium media due a nutrient depleted environment meaning they are likely to display similar metabolic activities to those cells causing infection *in vivo*. Also, the bacteria are less likely to have major adverse effects on the epithelial cells *in vitro* that may occur if the bacteria were growing optimally in artificial broth and over-expressing many harmful virulence factors.

Completely differentiated CF bronchial epithelial cells grown under an air/liquid interface and pre-treated with LXA_4 prevented the rapid invasion of PAO1 in our *in vitro* model compared to cells that were untreated. Moreover, this delay in invasion was almost abolished when the epithelia was also treated with Boc-2, a potent inhibitor of the FPR2 receptor. This result once again proves that ALX/FPR2 receptor is a mediator of the physiological effects induced by LXA_4 on bronchial epithelium as previously reported (128, 129, 141).

We also investigated the ability of two clinical isolates to invade both a CF and a non-CF cell line *in vitro* that had either been pre-treated with LXA_4 or that remained untreated. The delay of invasion by *P. aeruginosa*1 was constant for up to 4 h post inoculation of NuLi-1 and CuFi-1 cells when treated 24 h prior to inoculation with

LXA_4. However, it should be noted there is some debate as to whether *P. aeruginosa* actually invades epithelial cells in CF lung. It is generally believed that *P.aeruginosa* favours the formation of biofilm within the CF lung (144, 145) but there are also a number of reports that demonstrate that *P. aeruginosa* can also invade epithelial cells in early stages of infection (146-150). *Garci-Medina et al* 2005 showed that *P. aeruginosa* invades epithelial cells in an attempt to evade the host defences while also developing resistance within the epithelial cell to be used for later infections (151). The amount of *P. aeruginosa* 2 invading epithelial cells is increased compared to *P. aeruginosa*1. *P. aeruginosa* 2 was also isolated from a different CF patient that was chronically infected, the ability to invade epithelial cells on greater scale needs further analysis with possible phenotyping of both strains before and after treatment with LXA_4.

The potential for LXA_4 to delay the invasion of non-CF and CF bronchial epithelium *by P. aeruginosa* warrants further investigations. For example, firstly it would be necessary to investigate further if LXA_4 can inhibit the growth or motility of *P. aeruginosa* and secondly whether LXA_4 increases the concentration of antibacterial peptides released by epithelial cells. As previously mentioned, LXA_4 increases tight junction formation in non-CF bronchial epithelial cells but the effect in CF cells has not been examined. The increase in tight junctions induced by LXA_4 may play a vital role in mediating the invasion of epithelial cells by *P. aeruginosa*. These questions will be further explored in Chapter VI.

The results presented here demonstrate for the first time that the endogenous lipid mediator LXA_4, not only mediates inflammation but increases ASL height and epithelial repair but may also play a role in protecting bronchial epithelial cells from invasion by pathogens such as *P. aeruginosa,* indicating a novel role for LXA_4 in the treatment of CF airway disease.

Chapter VI - Lipoxin A_4 increases tight junction barrier function to protect bronchial epithelium cells from invasion by *Pseudomonas aeruginosa*

6.1. Introduction

As described in Chapter VI, LXA_4 can delay the in the invasion of CF bronchial epithelial cells by the pathogen *P. aerugionsa*. There are many potential mechanisms that may be involved in delaying the invasion of CF bronchial epithelial cells. One mechanism of particular interest to our laboratory, involves the effect of LXA_4 on TJ formation (120). Our recent findings suggest that a decrease in the availability of LXA_4 in the CF lungs may be associated with a decrease in the levels of TJ formation often seen in this condition at the bronchial epithelial layer. This in turn may increase the bacterial invasion of bronchial epithelium.

ZO-1 proteins form an intrinsic part of the TJs. In ZO-1 depleted cells, barrier-forming proteins, such as claudin and occludin, are unable to assemble into the characteristic strands normally found at TJ sites. This in turn results in the disorganisation and disruption of TJs formation and function (152). Recent studies also suggest that ZO proteins are required for the assembly and function of adherence junctions (AJ). ZO-1 and ZO-2 are recruited to the AJs that assemble following epithelial cell-cell contact and only segregate from AJs into growing TJs as cells polarise (153). It is also now recognised that ZO proteins are required not only for TJ assembly but also for regulating the organisation and functional activity of apical cytoskeleton that are relevant for cellular organisation, epithelial repair and cell morphogenesis (89, 154).

During the repair of epithelial wounds, tight junctions are disrupted not only in the wounded area but also between uninjured cells at some distance from the area of damage (142, 155). Hence, the loss of ZO-1 proteins will render the cell accessible to invasion by opportunistic pathogens that can circumvent the apical membrane and invade epithelial cells basolaterally. This disruption in TJ may account for the enhanced internalisation of *P. aeruginosa* that has been seen when epithelial barrier integrity is lost (142).

LXA_4 increases ASL height, increases epithelial repair in CFBE (135, 141) and in non-CF bronchial epithelial cells, LXA_4 rapidly increased expression of ZO-1 to the apical membrane (120), however, the role of LXA_4 on ZO-1, protein expression, localisation and integrity during bacterial infection has not yet been tested in CF airway epithelial cells.

6.1.2. Aim

The aim of this chapter was to investigate further the ability of LXA_4 to increase tight junction formation and explore the role it plays in protecting non-CF and CF bronchial epithelial cells from invasion by *P. aeruginos* as demonstrated in Figure 6.1.

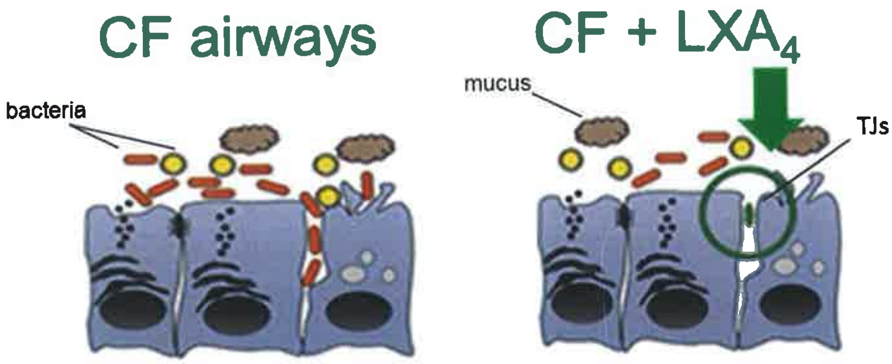


Figure 6.1: LXA_4 protects CF bronchial epithelial cells by increasing tight junctions. In CF airways as a result of the persistent infection and inflammation the epithelial protective barrier is damaged leading to an increase in bacterial invasion and transmigration. It is proposed that LXA_4 increases tight junctions (TJs) in CF bronchial epithelium cells which could delay the invasion and transmigration of bacteria across the bronchial epithelial barrier of the CF lung.

6.2. Specific Methodologies

6.2.1. Determing the growth rates of *P. aeruginosa*1 and *P. aeruginosa*2 *in vitro*

*P. aeruginosa*1 and *P. aeruginosa*2 were grown in LB broth overnight. From this overnight culture, a 1:50 dilution was prepared in fresh sterile LB Broth, 50/50 Bronchial Epithelium media, or 50/50 Bronchial Epithelium media with LXA_4 (1 nM) and incubated at 37^0C with shaking. From these growth conditions the OD_{600} was determined and CFU/ml calculated. Briefly, the OD_{600} of *P. aeruginosa*1 and *P. aeruginosa*2 in the differing growth mediums was recorded every 1.5 h until growth had reached stationary phase. The CFU/ ml was also determined at 1.5 h intervals by serial broth dilution and culture on LB agar. Agar plates for each dilution were incubated at 37^0C overnight. The number of CFU/ml was calculated and recorded until stationary phase of growth was achieved by optical density.

6.2.2. Motility assay

Motility of *P. aeruginosa*1 was determined using 0.3% LB agar. Bacteria were equalised to OD_{600} 0.2. From this equalised bacterial suspension 20 µl was inoculated into the centre of a LB agar (0.3% w/v) plate. Plates were incubated at 37^0C for 24 h. Images were recorded for each growth condition using the Gel-Doc-it, UVP imaging system. Image J was used to determine the total area of the LB agar plate and the total area of motility. Motility of *P. aeruginosa* was determined as a percentage of the total area of the LB agar plate.

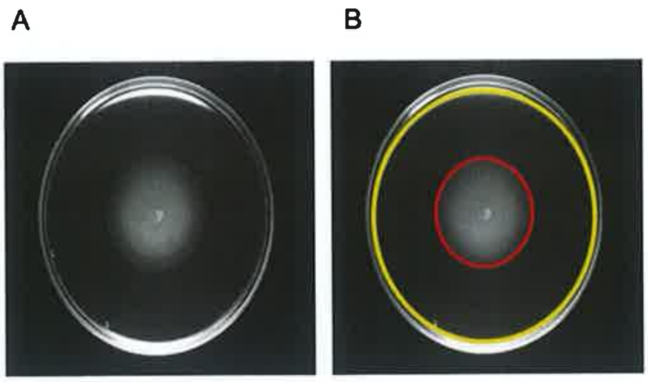


Figure 6.2: Determining the degree of *P. aeruginosa* motility upon *in vitro* culture. (**A**) Growth of *P. aeruginosa* demonstrating motility on an LB agar plate. (**B**) The area of motility was recorded and the percentage of motility was determined using Image J software. The halo of growth (defined by red circle) was reported as a percentage of the entire area of agar plate (defined by yellow circle).

6.2.3. LL-37 ELISA

An LL-37 ELISA (Hycult Biotech) was used as an *in vitro* quantitative determination of the concentration human LL-37 antimicrobial peptide released from NuLi-1 and CuFi-1 cells lines upon stimulation with LXA_4 (1 nM). The assay was performed according to the manufacturer's instructions.

6.2.4. ZO-1 Expression

ZO-1 protein expression was determined in non-CF and CF bronchial epithelium using Immunofluoresecne and Western Blotting. For Immunofluorescence experiments, epithelia where grown to fully differentiated, polarised epithelia and on transwell supports as previously described (sections 2.2. and 2.3. of the Materials and Methods). Cells were treated and fixed with 4% paraformaldehyde in PBS, permeabilised in 0.4% (v/v) Triton-X 100 diluted in PBS (Gibco, UK) and blocked in 3% (v/v) BSA (Sigma). Cells were then exposed to a 1:1000 dilution of mouse anti-ZO-1 primary antibody (Molecular Probes) and a 1:500 dilution of the secondary antibody Alexflour 488 conjugated anti-mouse (Invitrogen, NZ). Cells were set in Vectashield Hard Set containing DAPI (Vector Labs, UK) overnight at 4^0C. Immunofluorescence was observed using confocal microscopy (Ziess) with Z-Stacks performed on each slide at 3-4 randomised points.

For Western Blotting (protocol as described in section 2.7. of the materials and methods), transferred protein was blocked for 1 h at room temperature. Anti-ZO-1 primary antibody was used at 1:1000 dilution in milk-PBST (0.01% (v/v) overnight at 4^0C). A 1:5000 dilution of anti-mouse linked to HRP (Cell Signalling, USA) in 5% milk-PBST 0.01% (w/v) was used as a secondary antibody. Protein was observed using SuperSignal - West Dura chemilluminessece (Thermo Scientific). As a loading control mouse anti-α-tubulin (Abcam) was used at a dilution of 1:5000 in milk-PBST 0.01% (w/v) and anti-mouse linked to HRP as secondary antibody. Image J software was used to normalise protein levels.

6.2.5. Visualisation of invasion

NuLi-1 and CuFi-1 cells were grown to fully differentiated polarised epithelium on transwell supports. Stimulated cells were treated with LXA_4 (1 nM) 24 h prior to infection with *P. aeruginosa*1. *P. aeruginosa*1 was grown in LB broth overnight at 37^0C shaking. A 1:20 dilution of the bacterial culture was added to 50/50 bronchial epithelium media and allowed to grow to log phase and optical density of approximately OD_{600} 0.6. Bacteria were equalised to an optical density OD_{600} 0.2 and then re-suspended in 1 ml of fresh 50/50 bronchial epithelium media at a temperature of 37^0C. From this, 25 µl of bacterial solution was added apically to NuLi-1 or CuFi-1 cells for a 1 h or 3 h depending on experiment. Once the required inoculation time had been reached the epithelial cells were washed apically with PBS three times. Cells were fixed with 4% paraformaldehyde in PBS for 10 min. Cells were washed in PBS a further three times and permeabilised with 0.25% TritonX-100 for 10 min at room temperature. Cells were washed again in PBS three times. Cells were blocked in PBS containing 10% (v/v) goat serum (Sigma) and 1% (w/v) BSA for 1 h at room temperature. Cells were incubated simultaneously with primary anti-rabbit *P. aeruginosa* (see section 6.2.4.1) 1/50 dilution and mouse primary anti-ZO-1 at a dilution of 1/500 in blocking buffer at room temperature for 1 h. Cells were washed with PBS three times and incubated with a 1:500 diluted solution of secondary anti-mouse AlexaFlour 488 for 1 h at room temperature in blocking buffer. Cells were then washed again with PBS three times and incubated with a 1:500 diluted solution of secondary anti-rabbit AlexaFlour 594 (Invitrogen, NZ) (at room temperature for 1 h in blocking buffer).

Cells were washed a final time with PBS three times before being set in Vectashield Hard Set containing DAPI (Vector Labs, UK). Slides were allowed to set overnight before immunofluorescence was observed using confocal microscopy (Ziess). Z-Stacksing was performed on each slide at 3-4 randomised points.

6.2.5.1. Synthesis of *P. aeruginosa* antibody

The *P. aeruginsoa* polyclonal antibody was developed by Dr, M. Clyne and raised in New Zealand white female rabbits against *P. aeruginosa* strain PAO1. *P. aeruginosa* strain PAO1 was cultured in LB broth for 18 h at 37^{0}C with shaking. Bacteria were washed three times in PBS and centrifuged (6000 x g) for 10 min. The resulting pellet was re-suspended in PBS containing formaldehyde (2% (v/v)) at a concentration of 1 g (wet weight) per 100 ml of suspension. Bacterial suspensions were pooled, emulsified with Freunds Complete Adjuvant and used to inoculate two rabbits subcutaneously using 1 ml volumes. Two weeks later, a fresh emulsion was prepared in Freunds Incomplete Adjuvant and inoculated at fresh sites. Test bleeds were taken two weeks after initial inoculation to assess antibody production. Rabbits were exsanguinated and the blood was allowed to clot at room temperature. The serum was removed and stored at -20^{0}C.

6.2.6. Data analysis

The number of inserts used refers to the n number. NuLi-1, CuFi-1 experiments were repeated on 3 different cell passages under the same conditions. Primary bronchial cells were obtained from children under 6 years of age (patient numbers HCFBE90, HCFBE107 and SC004CB3; see appendix). Data is presented as mean ± S.E.M. of n experiments. Statistical significance was obtained using a Student T test and one way ANOVA test. Comparisons within groups were made using the post hoc test, Newman-Keuls. In all tests a P value ≤ 0.05 was deemed significant. All statistical analysis was carried out by GraphPad Prism.

6.3. Results

6.3.1. LXA_4 does not affect the growth of *P. aeruginosa*

The ability of *P. aeruginosa* to invade epithelial cells was hindered in the presence of LXA_4. Therefore the effect of LXA_4, alone, on growth of *P. aeruginosa* was investigated. Both clinical strains of *P. aeruginosa* were grown with or without the presence of LXA_4 (1 nM). *P. aeruginosa*1 and *P. aeruginosa*2 was grown in broth as a control growth condition, 50/50 bronchial epithelium media and 50/50 bronchial epithelium media supplemented with LXA_4 (1 nM). There was no difference in optical density and CFU/ml of *P. aeruginosa*1 when the isolate was grown in the presence of LXA_4 (1 nM) (Figure 6.3. (A) and (B)). The growth of *P. aeruginosa*2 was also not inhibited in the presence of LXA_4 (1nM) (Figure. 6.3. (C) and (D)). It would appear that LXA_4 alone does not inhibit the growth of *P. aeruginosa* alone.

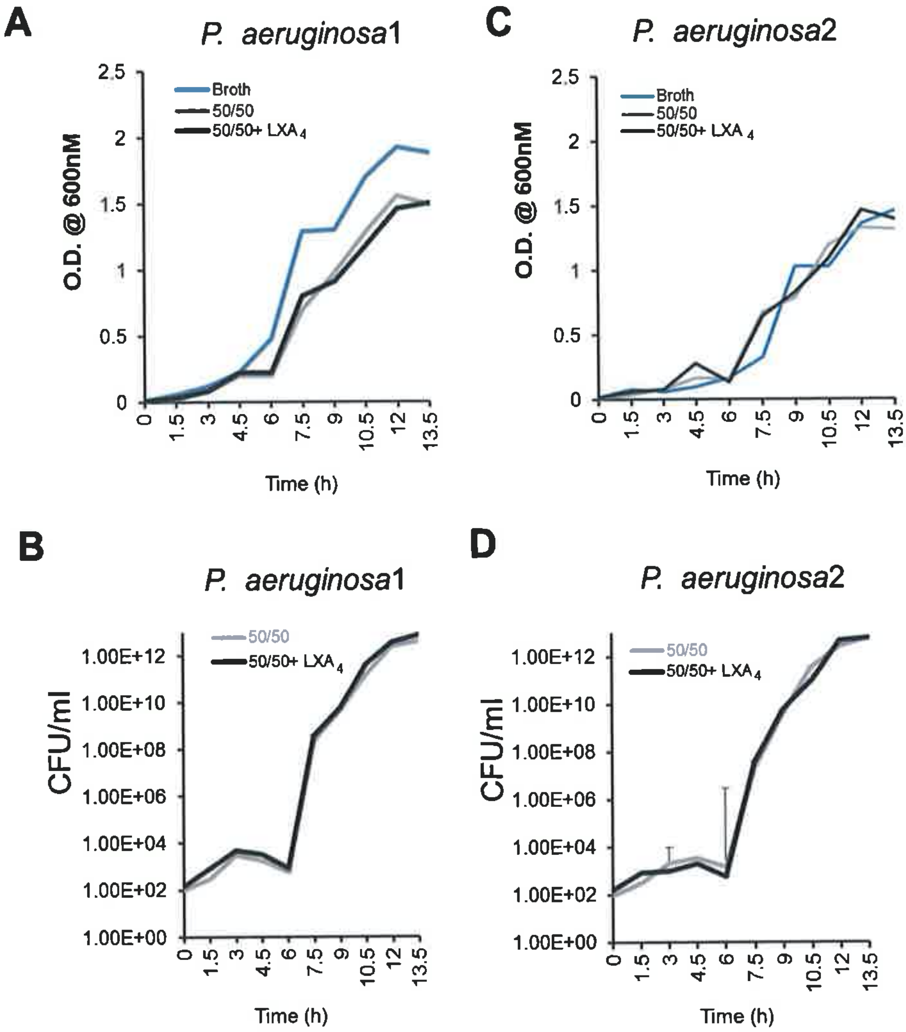


Figure 6.3: LXA_4 did not affect the growth of *P. aeruginosa*.

There was no difference in growth of *P. aeruginosa*1 and *P. aeruginosa*2 when grown in 50/50 bronchial epithelium media with or without the presence of LXA_4 (1nM) over 13.5 h when measured by optical density or number of CFU/ml (**A-D**)

6.3.2. The stable analogue of LXA_4, TA39, does not inhibit the motility of *P. aeruginsa* Clinical Strain 1

P. aeruginosa has the ability to move by rotating the monotrichous polar flagellum in aqueous environments. Motility of *P. aeruginosasa*1 was examined in the presence of LXA_4 alone using 0.3% motility LB Agar plates when grown in LB broth, 50/50 bronchial epithelium media, 50/50 + 100nM Ta39, 50/50 + 1nM TA39 and EtOH as a vehicle control. There was no significant difference in motility of *P. aeruginosa*1 grown in broth (24.58% ± 1.17, n=7), 50/50 (19.94% ± 1.9, n=7), 50/50 + 100nM Ta39 (23.87% ± 3.14, n=4), 50/50 + 1nM TA39 (23.91% ± 2.0, n=4) or EtOH (21.6% ± 0.4, n=4) (Figure: 6.4). LXA_4's stable analogue alone does not affect the motility of *P. aeruginosa*1.

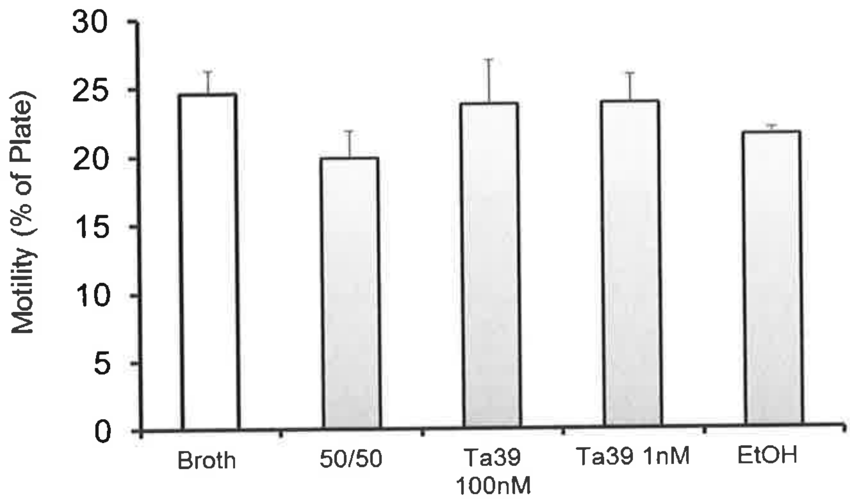


Figure 6.4: The stable analogue of LXA_4, TA39, did not inhibit the motility of *P. aeruginosa*. Motility of *P. aeruginosa*1 was measured when grown in LB Broth, 50/50 bronchial epithelium media, 50/50 media plus TA39 (100 nM), 50/50 media plus TA39 (1 nm) and EtOH as a vehicle control. Growth of motility was measured as a percentage of the total Agar plate. LB Broth (n=7), 50/50 bronchial epithelium media (n=7), 50/50 + 100nM TA39 (100 nM) (n=4), 50/50 + 1nM TA39 (1 nM) (n=4). There was no significant difference in motility of *P. aeruginosa*1 when bacteria were grown in the presence of LXA_4 stable analogue Ta39.

6.3.3. *P. aeruginosa* motility is not inhibited following exposure to CF bronchial epithelial cells treated with LXA_4

The ability of LXA_4 to delay the invasion of the airway epithelium by *P. aeruginosa* by inhibiting motility was further analysed in the presence of epithelial cells. A motility assay involving *P. aeruginosa*1 was performed using CF bronchial epithelial cells that had been for treated for 24 h, prior to inoculation, with LXA_4 (1nM). No significant decrease in motility was observed when bacteria were retrieved from the apical membrane of CF bronchial epithelial cells pre-treated with LXA_4 (1 nM) (11.02% ± 3.65, n=3) compared to non-stimulated epithelial cells (12.44% ± 2.12, n=3). Also, no difference in motility of *P. aeruginosa*1 can be seen following transmigration to the basolateral compartment between non-stimulated (12.19% ± 2.36, n=3) cells and CF bronchial epithelial cells stimulated with LXA_4 (1nM) (13.51% ± 3.42). There was also no significant difference in the motility of bacteria that had been retrieved from the apical membrane and bacteria that had transmigrated to the apical compartment. It appears that the pre-treatment of CF bronchial epithelial cells with LXA_4 (1 nM) does not affect the motility of *P. aeruginosa*1 (Figure 6.5. (A)).

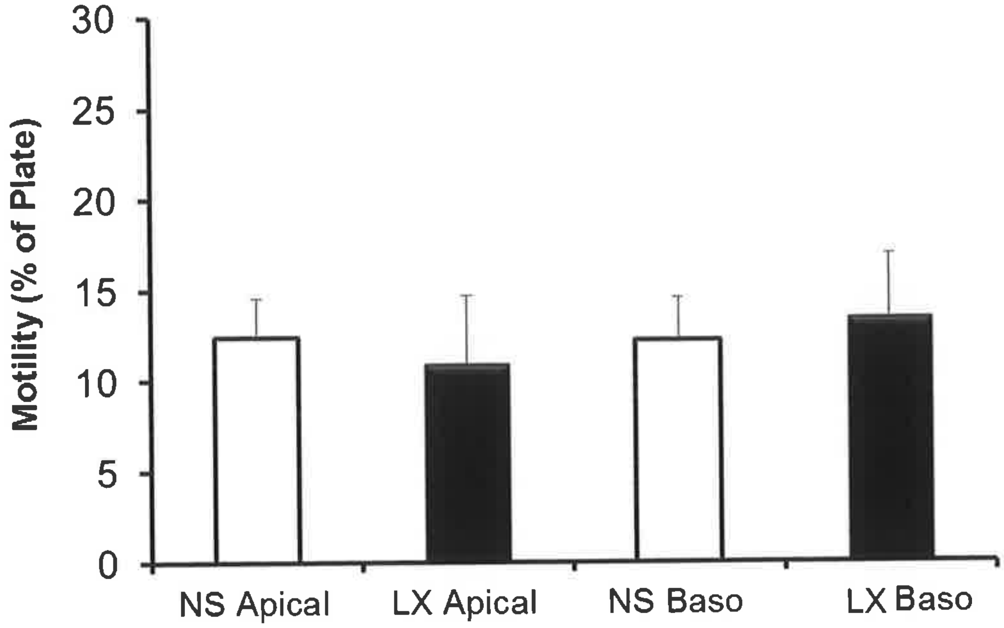


Figure 6.5: Exposure of *P. aeruginosa* to CF bronchial epithelial cells treated with LXA_4 did not inhibit *P. aeruginosa* motility over time. Exposure of CF bronchial epithelial cells to LXA_4 (1 nM) 24 h prior to inoculation with *P. aeruginosa*1 did not affect the motility of bacteria collected 1 h post inoculation compared to non-stimulated (NS) epithelial cells. The percentage motility was measured from *P. aeruginosa*1 collected from the apical membrane or bacteria that had transmigrated to the basolateral (Baso) compartment (n=3).

6.3.4. Treatment of NuLi-1 cells with LXA_4 does not inhibit the motility of *P. aeruginosa* 1-3 h post inoculation

The effect on motility of *P. aeruginosa*1 by LXA_4 (1 nM) over 3 h was also examined to investigate if LXA_4 can inhibit motility of the bacterial cells over time. There was no significant difference in the motility of *P. aeruginosa*1 retrieved form the apical membrane of NuLi-1 cells pre-treated with LXA_4 (1 nM) at 1 h post inoculation (16.15% ± 0.3, n=2), 2 h post inoculation (10.21% ± 2.2, n=2) and 3 h post inoculation (12.31% ± 0.16, n=2) (Figure 6.6. (A)). Bacteria that had transmigrated through the epithelial barrier to the basolateral membrane also showed no significant change in motility at 1 h post inoculation (18.24% ± 4.2, n=3) and at 2 h post inoculation (25.71% ± 4.2, n=3) (Figure 6.6. (B)). However, an increase in motility can be seen in bacterial cells that had transmigrated to the basolateral compartment compared to those bacterial cells that had been retrieved from the apical membrane. However, this finding was not significant.

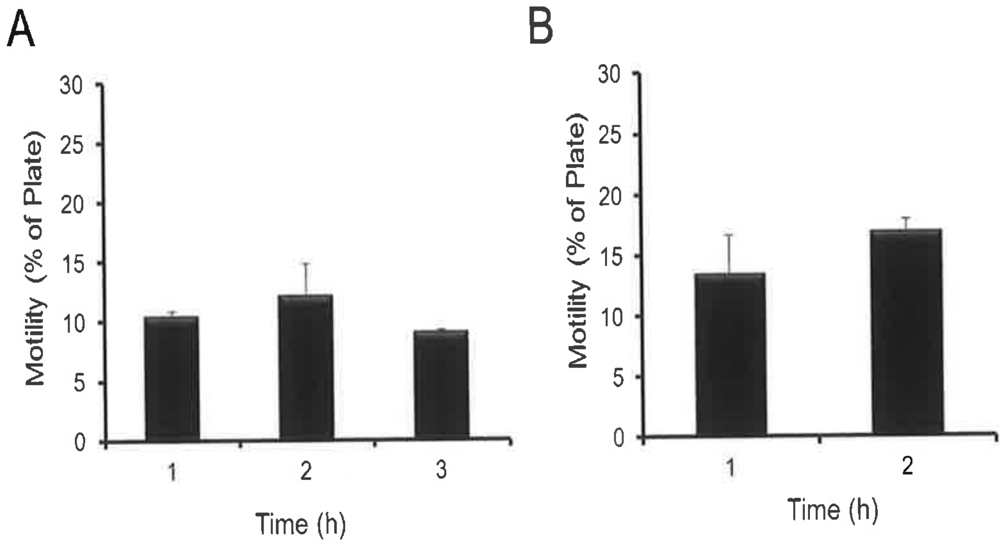


Figure 6.6: Treatment of NuLi-1 cells with LXA_4 did not affect motility of *P. aeruginosa* over 3 hours. (A) The motility of *P. aeruginosa*1 was not affected following exposure of NuLi-1 cells to LXA_4 (1 nM) 24 h prior to inoculation with the bacterial cells. Bacteria were collected from the apical membrane from 1 -3 h post inoculation (n=2). (**B**) Motility of *P. aeruginosa*1 following transmigration to the basolateral compartment. Again, no significant difference in motility of bacterium can be seen 1 h and 3 h post inoculation (n=2).

6.3.5. Treatment of CuFi-1 cells with LXA_4 does not affect motility of *P. aeruginosa* over a 3 h period

The CF cell line (CuFi-1) was also pre-treated with LXA_4 (1 nM) and subjected to inoculation with *P. aeruginosa*1 to investigate if a difference in bacterial cell motility could be observed over a 3 h period. Our findings indicate that there was no significant difference observed in the motility of bacterial cells retrieved from the apical membrane 1 h post-inoculation (10.57% ± 0.2, n=3), 2 h post-inoculation (12.31% ± 2.51, n=3) and 3 h post-inoculation (9.16% ± 0.05, n=3) (Figure 6.7. (A)). Similarly, No significant increase in motility of the bacterial cells was observed 1 h post-inoculation at the basolateral compartment (13.59% ± 3.0, n=3) (Figure 6.7. (B)). However, Increased motility could be seen in bacterial cells that had transmigrated to the basolateral compartment 2 h post-inoculation (17.06% ± 3.08, n=3).

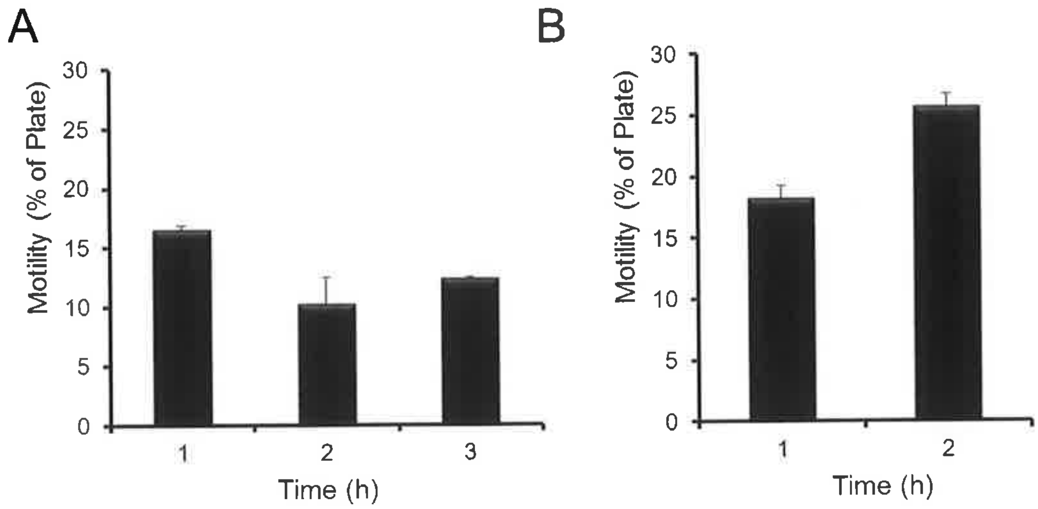


Figure 6.7: Treatment of CuFi-1 cells with LXA_4 did not inhibit the motility of *P. aeruginosa*. The CF bronchial epithelium cell line, CuFi-1, was exposed to LXA_4 (1 nM) 24 h prior to inoculation with *P. aeruginosa*1. Motility of *P. aeruginosa*1 was measured as a percentage of the total agar plate from bacteria exposed apically to CuFi-1 cells or treated with LXA_4 (1 nM). (**A**) No significance difference in *P.aeruginosa*1 motility can be seen from bacteria collected from the apical membrane from 1-3 h post inoculation (n=2). (**B**) An increase in motility can be seen in *P. aeruginosa*1 cells that transmigrated to the basolateral compartment after 2 h post inoculation but this was not significant (n=2).

6.3.6. LXA_4 has no effect on the motility of *P. aeruginosa* cells that had invaded NuLi-1 and CuFi-1 cells

As previously shown, the treatment of bronchial epithelial cells with LXA_4 decreases the ability of *P. aeruginosa* to invade them. The motility of *P. aeruginosa*1 cells, 3 h post-inoculation, which had invaded NuLi-1 and CuFi-1 epithelial cells pre-treated with LXA_4 (1 nM) was also investigated. In Figure 6.8 (A), it can be seen that the treatment of NuLi-1 cells with LXA_4 (1nM), prior to inoculation with *P. aeruginosa*1, causes no difference in the motility of the bacteria that had invaded the epithelial cells 3 h post inoculation (31.01% ± 1.3, n=3) compared to non-stimulated cells (29.15% ± 4.36, n=3). The same is also true for CuFi-1 cells pre–treated with LXA_4. The motility of the bacterial cells that had invaded CuFi-1 cells pre-treated with LXA_4 does not decrease (29.05% ± 3.2, n=3) compared to non-stimulated epithelial cells (29.05% ± 3.2, n=3) (Figure 6.8. (B)). Together, these data suggest that LXA_4 does not affect the motility of *P. aerugionsa* and hence its ability to invade epithelial cells.

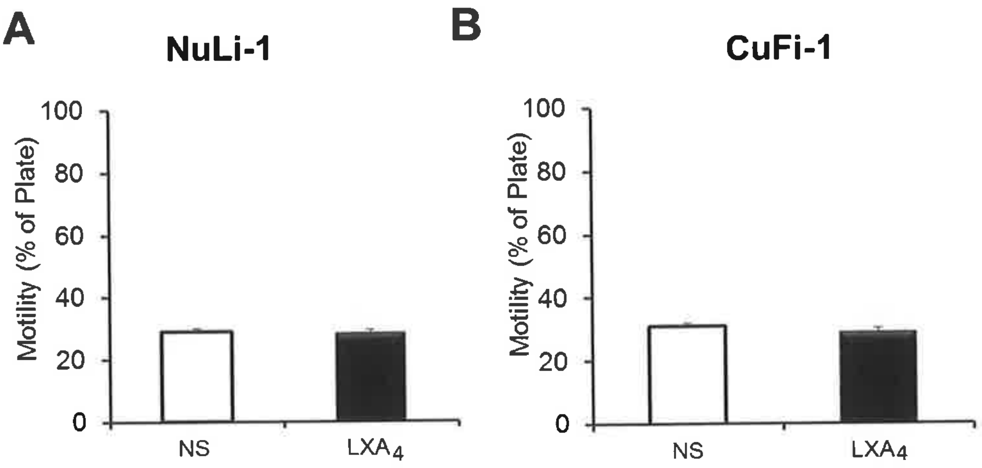


Figure 6.8: LXA_4 had no effect on motility of *P. aeruginosa* cells that had invaded NuLi-1 and CuFi-1 cells. Bacterial cells that had invaded NuLi-1 and CuFi-1 cells were recovered using Triton-X100 (0.4% v/v) lysis 3h post inoculation with *P.aeruginosa*1. (**A**) The motility of the recovered bacteria was assessed. Here no significant decrease in motility of *P. aeruginosa*1 cells can be seen following invasion of NuLi-1 cells treated with LXA_4 (1 nM) or left untreated 24 h prior to inoculation (n=3). (**B**) Similarly, no significant effect on *P.aeruginosa1* motility can be seen following the invasion of CuFi-1 treated with LXA_4 (1 nM) or left untreated 24h prior to inoculation (n=3).

6.3.7. LXA_4 does not increase LL-37 release from NuLi-1 and CuFi-1 cells

LXA_4 does not have an effect on cell motility but does significantly decrease the number of invading *P. aeruginosa* cells into non-CF and CF bronchial epithelial cells. It is proposed the effect of LXA_4 on bacterial invasion is mediated by the antimicrobial peptide LL-37 release from epithelial cells. To investigate this hypothesis, NuLi-1 and CuFi-1 cells were firstly treated with LXA_4 (1 nM) for 1 h and then LL-37 levels were determined at the apical and basolateral layers and compared to the control of non-stimulated cells. There was no difference in the levels of LL-37 released apically from NuLi-1 cells following treatment with LXA_4 (0.29 ng/ml ± 0.006, n=3) compared to untreated cells (0.28ng/ml ± 0.005, n=3) (Figure 6.9. (A)). No difference in LL-37 levels at the apical side of CuFi-1 cells is also observed following treatment with LXA_4 (0.31ng/ml ± 0.006, n=3) compared to non-stimulated CuFi-1 cells (0.31ng/ml ± 0.01, n=3) (Figure 6.9. (B)). Detection of LL-37 released basolaterally was below the threshold of the ELISA and considered 0 µg/ml. These data indicate that LXA_4 does not affect the concentration of LL-37 released from NuLi-1 and CuFi-1 cells alone.

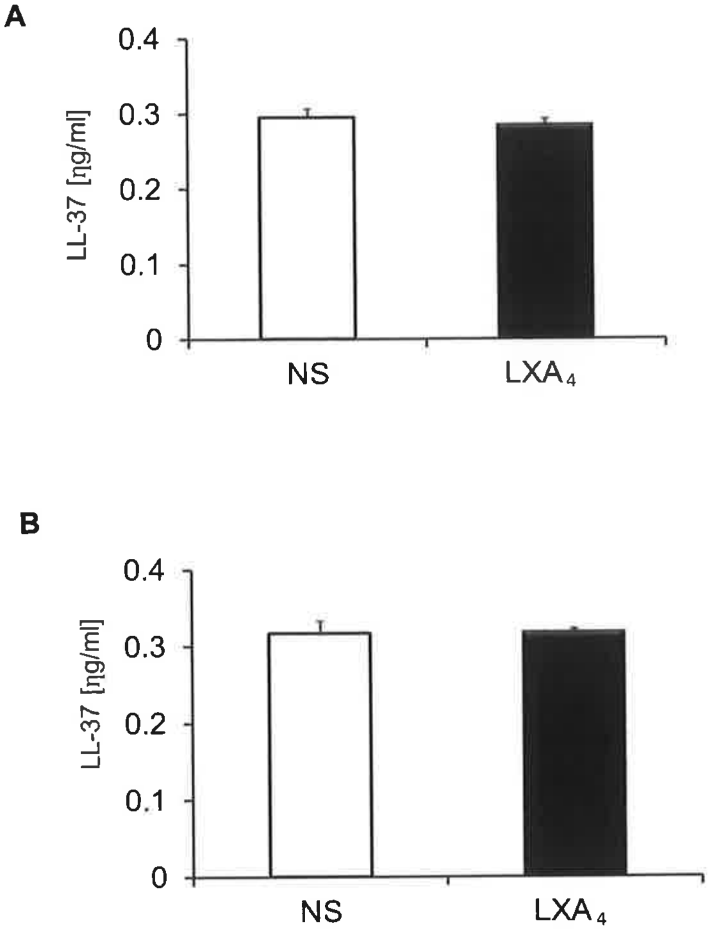


Figure 6.9: LXA_4 did not induce secretion of the antimicrobial peptide LL-37 from NuLi-1 and CuFi-1 cells. NuLi-1 (**A**) and CuLi-1 (**B**) were treated with LXA_4 (1 nm) for 24 h prior to collection of cell supernatants. LL-37 levels were quantified by ELISA. (**A**) No significant difference in the concentration of LL-37 released apically by NuLi-1 cells can be seen following treatment with LXA_4 (1 nM) (n=3) compared to non-stimulated conditions (NS) (n=3). (B) There was also, no significant difference in the concentration of LL-37 released apically from CuFi-1 cells treated with LXA_4 (1 nM) (n=3) compared to non-stimulated (NS) epithelial cells (n=3).

6.3.8: LXA_4 does not affect the concentration of LL-37 released by NuLi-1 and CuFi-1 cells stimulated with *P. aeruginosa* cells

The inability of LXA_4 to induce LL-37 release from NuL1-1 and CuLi-1 epithelial cells alone was further analysed following stimulation with live *P. aeruginosa*1. Again, there was no significant difference in the concentration of LL-37 released apically from NuLi-1 cells pre-treated with LXA_4 (1 nM) 24 h prior to inoculation with *P. aeruginosa*1 cells (0.21ng/ml ± 0.04, n=3) compared to non-stimulated epithelial cells (0.3ng/ml ± 0.01, n=3) (Figure 6.10. (A)). Similarly, no difference in the levels of LL-37 (0.28 ng/ml ± 0.01, n=3) released apically was seen in CuFi-1 cells, which had undergone the same treatment with LXA_4 (1nM) and stimulation with bacterial cells, compared to non-stimulated control cells (0.26ng/ml ± 0.002, n=3) (Figure 6.10. (B)). No production of LL-37 released to the basolateral compartment was seen. These findings suggest that LXA_4 does not affect LL-37 production from NuLi-1 and CuFI-1 cells in the presence of *P. aeruginosa*1 cells.

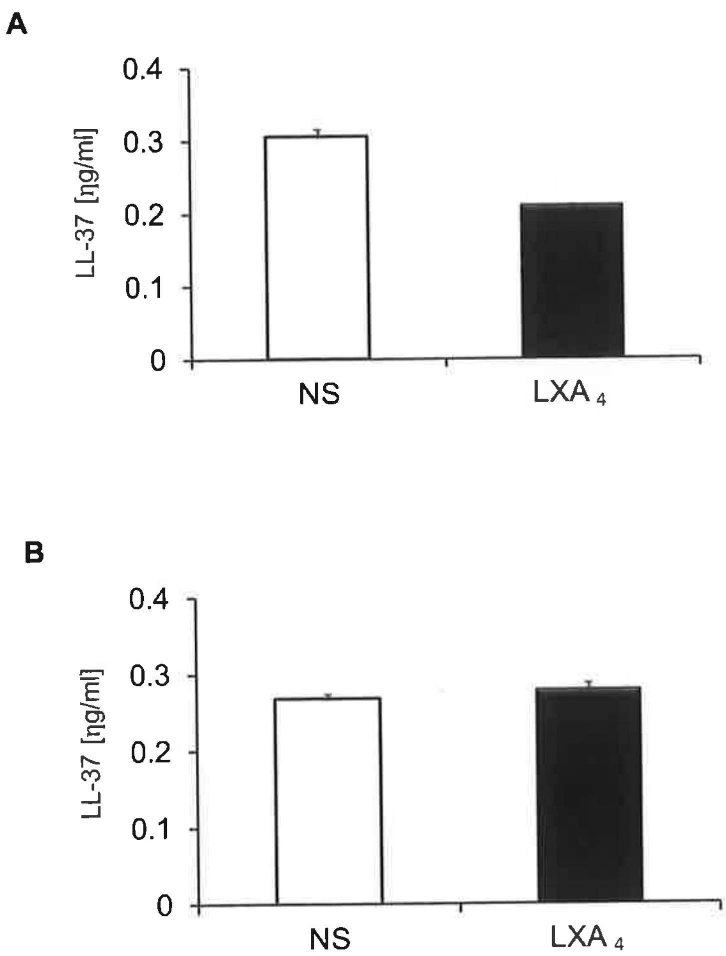


Figure 6.10: LXA$_4$ had no effect on LL-37 release by NuLi-1 and CuFi-1 cells inoculated with *P. aeruginosa*. NuLi-1 (**A**) and CuFi-1 (**B**) cell lines where treated with LXA$_4$ (1 nM) 24 h prior to stimulation with *P. aeruginosa*1. LL-37 levels were quantified by ELISA. (**A**) No significant difference in the levels of LL-37 released apically from NuLi-1 cells was observed following pre-treatment with LXA$_4$ compared to non-stimulated (NS) conditions following inoculation with *P. aeruginosa*1 (n=3). (**B**) Again, no significant difference in the levels of LL-37 released in the CF cell line (CuFi-1) was observed following pre-treatment with LXA$_4$ (1nM) compared to non-stimulated (NS) conditions 1 h post-stimulation with *P. aeruginosa*1 cells (n=3).

6.3.9. LXA_4 Increases ZO-1 expression in primary CF bronchial epithelial cells, NuLi-1 and CuFi-1 cells

As previously shown by our laboratory, LXA_4 increases ZO-1 expression (apical membrane) and protein levels in 16HBE14o- airway epithelial cells. However, the role that LXA_4 plays in tight junction formation in CF bronchial epithelial cell has not yet been investigated. Immunofluorescence and confocal microscopy on primary CF bronchial epithelial cells allows for visualisation of the ZO-1 protein upon stimulation with LXA_4. Primary CF bronchial cells display an increase in ZO-1 expression at the apical membrane following 30 min treatment of LXA_4 (1 nM) (Figure 6.11, (A)). ZO-1 protein levels where analysed by Western Blotting in two different CF patients (ΔF508/508) after stimulation with LXA_4 (1 nM) for 18 h. An increase in ZO-1 expression in both patient samples was clearly observed upon stimulation with LXA_4 (1 nM) (n=3), which was confirmed by densitometry (Figure 6.11. (B) and (C)). This increase in ZO-1 protein expression was also observed in the non-CF bronchial epithelium cell line (NuLi-1) and the CF bronchial epithelium cell line (CuFi-1). Both NuLi-1 and CuFi-1 cell lines treated with LXA_4 (1 nM) for 18 h resulted in a significant increase of ZO-1 protein expression compared to non-treated controls, which again was verified by densitometry (n=3, P **≤ 0.01, ANOVA) (Figure 6.11. (D) and (E)).

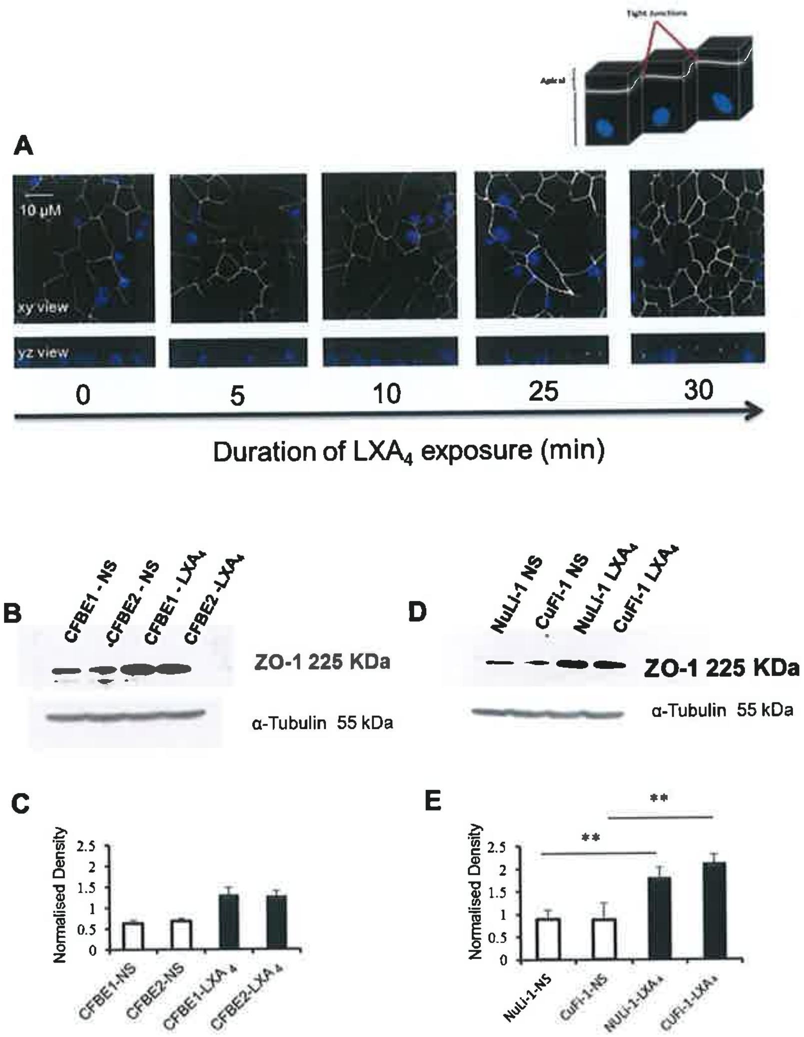


Figure 6.11: LXA_4 Increased ZO-1 Expression in CF bronchial epithelial cells cells. CF bronchial epithelial (CFBE) cells stimulated with LXA_4 (1 nM) display a rapid increase in expression of ZO-1 at TJ membranes from 0-30 min, which can be seen by immunofluorescence (**A**), an increase in ZO-1 is visible on xy view and also the yz view of the confocal images over time (white). (**B** and **C**) An increase in ZO-1 protein expression can be seen in primary CF bronchial epithelial cells from two different patients (CFBE1 and CFBE2) after 18 h stimulation with LXA_4 (1 nM). (**D** and **E**) ZO-1 expression also increased in the non-CF bronchial epithelium cell line NuLi-1 and CF bronchial epithelium cell line (CuFi-1) after an 18 h stimulation with LXA_4 (1 nM) (P **≤ 0.01).

6.3.10. LXA_4 increases ZO-1 protein expression in NuLi-1 and CuFi-1 cells post- inoculation with *P. aeruginosa*

NuLi-1 and CuFi-1 cells were pre-treated with LXA_4 (1nM) prior to infection with *P. aeruginosa*1. Protein was extracted from epithelial cells and Western Blot analysis was performed to determine the levels of ZO-1 protein expression pre- and post-infection with *P. aeruginosa*1. Pre-treatment of NuLi-1 cells with LXA_4 (1 nM) increases levels of ZO-1 protein expression. Inoculation of non-stimulated NuLi-1 cells with *P. aeruginosa*1 results in a decrease in Z0-1 protein expression at 1 h and 3 h post inoculation. However, following treatment with LXA_4 (1 nM), increased ZO-1 protein expression maintained. This increase was confirmed by optical densitometry of three different Western Blots (n=3, P *≤ 0.05, P **≤ 0.01) (Figure 6.12. (A and B)). CuFi-1 cells also treated with LXA_4 (1nM) resulted in an increase in ZO-1 protein expression compared to non-stimulated cells. This increase in ZO-1 protein expression was maintained 1 h and 3 h post inoculation with *P. aeruginosa*1 compared to non-treated CuFi-1 cells (n=3, P **≤ 0.01) (Figure 6.12. C and D).

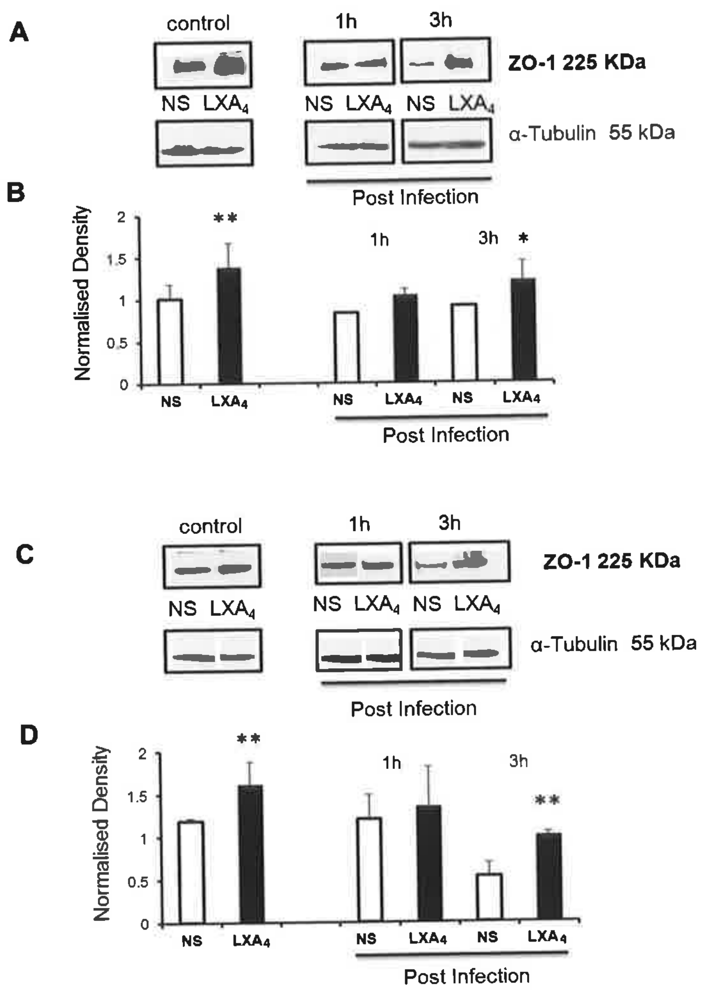


Figure 6.12: LXA_4 increased the protein expression of ZO-1 in NuLi-1 and CuFi-1 cells, which is sustained after infection with *P. aeruginosa*. (**A+C**) Under control conditions (no bacteria) there was an increase in ZO-1 protein levels when NuLi-1 and CuFi-1 cells where pre-treated with LXA_4 (1nM) compared to non-stimulated cells (NS). 1 h and 3 h post infection with *P. aeruginosa*1 resulted in a decrease of ZO-1 protein expression in both NuLi-1 and CuFi-1 cells. ZO-1 protein expression remained unchanged in NuLi-1and CuFi-1 cells treated with LXA_4 (1nM) 1 h and 3 h post inoculation. (**B+D**) The decrease in ZO-1 protein expression was confirmed by optical density in both NuLi-1 and CuFi-1 cells. (n=3, P *≤ 0.05, P **≤ 0.01, P ***≤ 0.001, T test)

6.3.11. LXA_4 increases expression of ZO-1 and protects tight junctions during infection of NuLi-1 cells

The invasion of NuLi-1 cells by *P. aeruginosa*1 was investigated further by using immunofluorescence and confocal microscopy. Images of the invasion where captured at 1 h and 3 h post infection of NuLi-1 cells pre-treated with LXA_4 (1 nM), or untreated as the basal control. NuLi-1 cells treated with LXA_4 (1 nM) 24 h prior to inoculation results in an increase of ZO-1 protein expression (white in Figure 6.13 (A and B)) at the apical membrane. Three hours post inoculation with *P. aeruginosa*1, a decrease in the levels of ZO-1 expression is observed at the apical membrane in non-stimulated cells compared to NuLi-1 cells pre-treated with LXA_4 (1nM). Cells pre-treated with LXA_4 (1 nM) show no sign of degradation or loss of ZO-1 protein expression, 3 h post inoculation. However, in non-stimulated cells, a clear decrease in ZO-1 expression can be seen from 1 h to 3 h post inoculation (Figure 6.13. (A and B)). At 1 h post inoculation, an increase in the ability of the bacteria (green) to transmigrate, or invade, epithelial cells can be seen in non-stimulated cells compared to NuLi-1 cells that were treated with LXA_4 (1nM). The images also suggest that there is also some degree of bacterial cell aggregation in non-stimulated epithelial cells compared to treated epithelial cells in Figure 6.13. (C) and (D) which is evident from the grouping of bacteria on the apical surface of non-treated cells.

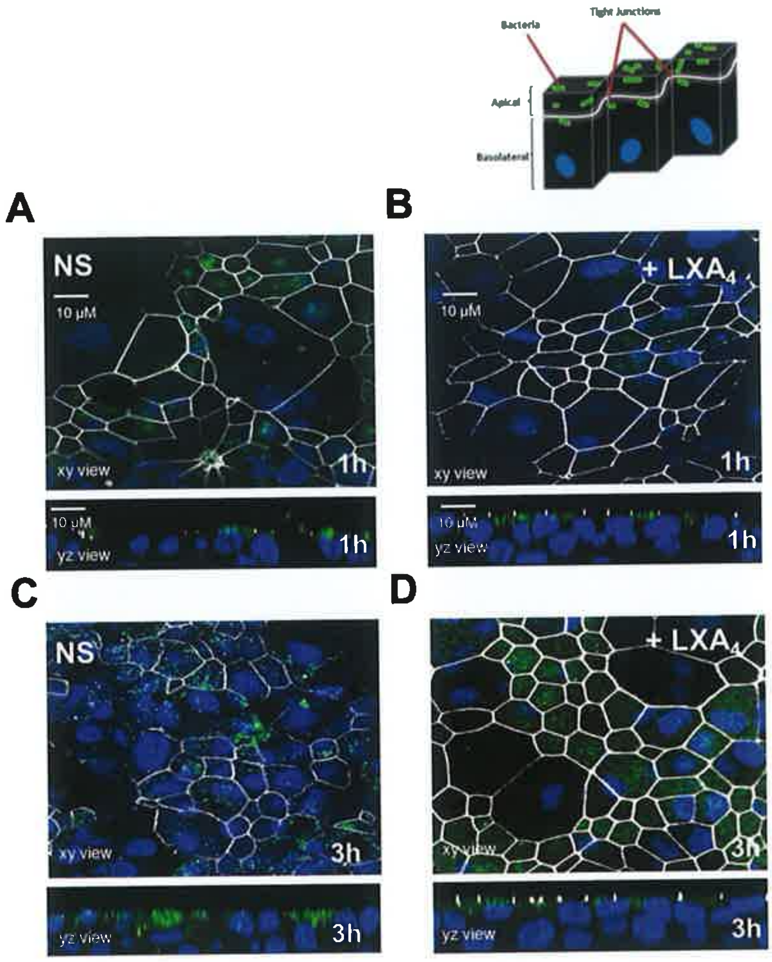


Figure 6.13: LXA_4 increased the expression of ZO-1 and protects tight junctions during infection of NuLi-1 cells. NuLi-1 cells were treated with LXA_4 (1 nM) 24 h prior to inoculation with *P. aeruginosa*1 (green) and non-stimulated cells as a control were fixed during infection at 1 h and 3 h post inoculation and stained for analysis by confocal microscopy. (**A** and **B** (xy view)) ZO-1 expression (white) is increased and sustained (1 h post-inocualtion) at the cell membrane when NuLi-1 cells are treated with LXA_4 prior to inoculation compared to basal (NS) levels. (**C** and **D** (xy view)) at 3 h post-infection a decrease in expression ZO-1 at cell membranes can be seen in untreated cells compared to NuLi-1 cells treated with LXA_4 (1nM).

6.3.12. LXA_4 increases and protects the expression of ZO-1 protein in CuFi-1 cells post infection

CuFi-1 cells were also analysed using immunofluorescence and confocal microscopy following invasion by *P. aeruginosa*1 at 1 h and 3 h post-inoculation. An increase in bacterial contact, invading, or transmigrating across the epithelial barrier in non-stimulated cells compared to cells treated with LXA_4 was seen at 1 h post-inoculation (Figure 6.14. (A and B)). ZO-1 expression is completely degraded in non-stimulated cells at 3 h post inoculation compared to those treated with LXA_4. This degradation of ZO-1 protein is clearly visible in both the xy and yz view of Figure 6.14. C and D. There is also an increase in bacterial aggregate formation on non-stimulated cells 3 h post-inoculation, compared to epithelial cells treated with LXA_4 (6.14. (C and D)). The decrease in Z0-1 expression visualised by immumfluoresece is directly correlated to the decrease in protein expression observed by Western Blot analysis (seen in Figure 6.11). Taken together these results suggest that LXA_4 may play a vital role in protecting the epithelial barrier during infection.

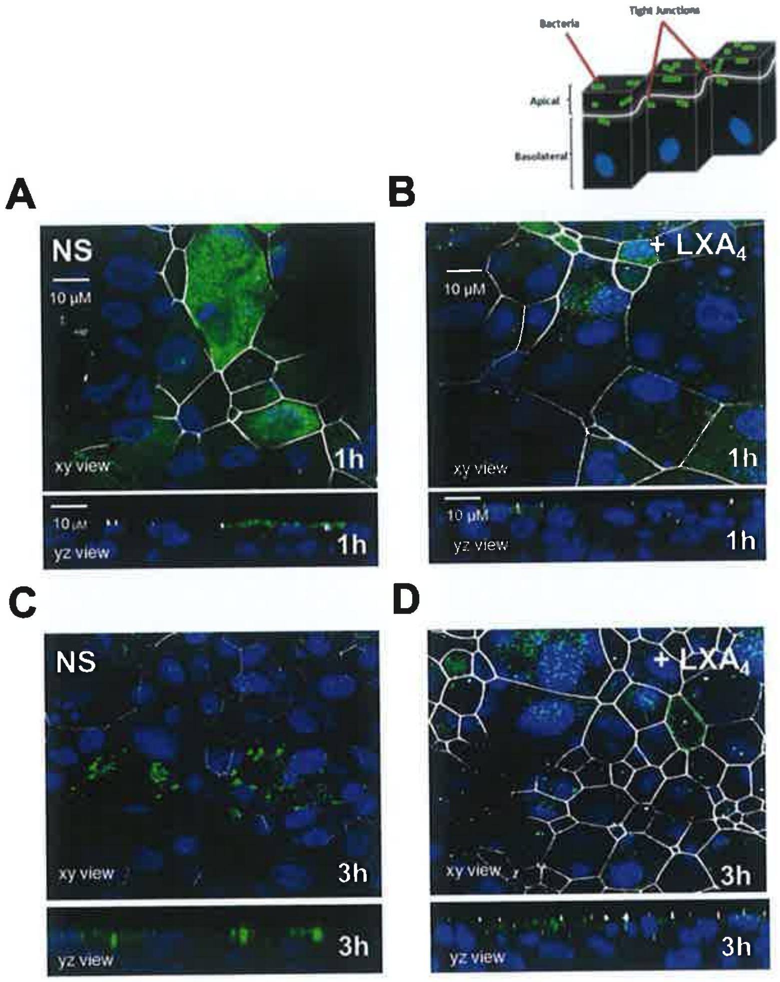


Figure 6.14: LXA_4 increased and protected the expression of ZO-1 during infection of CuFi-1 cells by *P. aeruginosa*1. CuFi-1 cells treated with LXA_4 (1 nM) 24 h prior to infection with *P. aeruginosa*1 (green) and non-stimulated cells as a control were fixed and stained for analysis by confocal microscopy. (**A** and **B** (xy view)) ZO-1 expression (white) is increased and sustained (1 h post-inoculation) at the cell membrane when CuFi-1 cells are treated with LXA_4 prior to inoculation compared to basal (NS) levels. (**C** and **D** (xy view)) At 3 h post inoculation a decrease in expression of ZO-1 can be seen at cell membranes in non-stimulated CuFi-1 cells compared to cells treated with LXA_4.

6.4. Discussion

The increase in bacterial burden in CF lungs is not fully understood. The work presented in this chapter provides evidence, in part; to further elucidate the interaction between *P. aeruginosa* and the epithelial layer in the CF lung, the subsequent innate immune responses and the role of LXA_4 in the pathogenesis of this disease. LXA_4 alone did not affect the growth or motility of *P. aeruginosa*, however, LXA_4 delayed the invasion of bronchial epithelia by bacteria via a cellular mechanism that is described, at least in part, in this chapter.

The human cathelicidin LL-37 has been reported to kill bacteria, destroy bacterial biofilms, and work as an antiviral agent when released from epithelial cells (156-158). LL-37 knockout mice showed an increase in susceptibility to bacterial infections and experience significantly higher morbidity and mortality (159, 160). LL-37 also plays a role in the recovery of tissue damage following the activation of the innate immune response to the invading pathogen during infection (161). It is for this reason that the role of LXA_4 in stimulating the release of LL-37 from epithelial cells was investigated. However, our findings showed that the concentration of LL-37 released from epithelial cells either basolaterally (result not shown) or apically did not alter upon stimulation with LXA_4. Further work would be required to completely rule out the role LL-37 plays in the inhibition of bacterial invasion induced by LXA_4. As LL-37 was found in the apical compartment and not released basolaterally, a positive control or real time – PCR data would be required to fully elucidate the roles of LXA_4 on LL-37 release and expression in the CF lung. Moreover, other reports have shown that LXA_4 can actually inhibit the pro-inflammatory effects of LL-37 by competitive binding of FPR2 receptor (162), but it should be noted that the ALX/FPR2 receptor may not be the only receptor for the LL-37 peptide (161).

The barrier function of airway epithelium is regulated by the apical intercellular junctional complex, which includes both tight and adherence junctions. AJ mediate cell-to-cell contact and promote formation of TJs that in turn regulate transportation of solutes and ions across epithelia (154). Under homeostasis, these intercellular apical junction complexes prevent inhaled pathogens, and other environmental insults, from injuring the airways. They also serve as signalling platforms that regulate gene expression, cell proliferation, and differentiation (89). It has been reported that sustained infection and inflammation affects these junction complexes by not only disrupting barrier function, but also by interfering with the normal repair and differentiation of airway epithelium. Infection with viruses or bacteria can cause transient disruption of AJ and TJs (163, 164). The sustained and prolonged host innate immune response typical of the CF lung may further prolong barrier dysfunction and irregularity. By using immunofluorescence and Western Blotting these data show, for the first time, that LXA_4 increases ZO-1 protein expression in CF bronchial epithelial cells and decreases the ability of *P. aeruginosa* to invade epithelial cells. This increase in TJ formation may decrease the sustained inflammation observed in the CF lung by increasing epithelial integrity and denying peripheral blood mononuclear cells an uncontrolled passage towards the site of infection.

The increase in ZO-1 expression in response to LXA_4 has previously been reported in the 16HBE14o bronchial epithelial cell line (120). To the best of our knowledge, this is the first time that an increase in ZO-1 has been observed upon stimulation with LXA_4 in bronchial epithelia from patients with CF (CuFi-1 cell line and CF primary culture). Rapid expression of the tight junction protein ZO-1 to the apical membrane in CF bronchial epithelium cells was visualised by immunofluorescence and this increase was sustained following 16 h stimulation with LXA_4. The increase in ZO-1 protein is sustained even after inoculation with the pathogen *P. aeruginosa*. Confocal microscopy was also used to visualise the invasion of epithelial cells by the bacteria and found a clear destruction of ZO-1 architecture in non-treated cells compared to epithelial cells treated with LXA_4. It has been shown that reduction in ZO-1 protein expression can result in an increase in bacterial burden (142, 143, 165). The delay in the invasion by *P.aeruginosa* reported in this work directly correlates to the increase in ZO-1 protein expression. It is also noted that *P. aeruginosa* preferentially invades and adheres to damaged epithelial cells (142, 143, 155, 166). Therefore, the decrease of ZO-1 protein expression in the CF lung, resulting from a decrease of LXA_4, may be responsible for the increased rate of infection by *P. aeruginosa*.

P. aeruginosa has the ability to secrete virulence factors disrupting TJs, which can increase the early infiltration of airway cells. These secretable factors include rhampholids (165), *P. aeruginosa* elastase (143), and exotoxin A (146). It may be that LXA_4 influences the expression of *P. aeruginosa* virulence factors by some undefined pathway at the microbial level, but further analysis examining virulence factor gene expression using Real Time-PCR would be required to answer this question. Further examination of bacterial genes at a transcriptional level from *P. aeruginosa* that have invaded non-stimulated and stimulated epithelial cells with LXA_4 would be worth considering. The ability of LXA_4 to alter the bacterial genome has not been reported as yet and would be an interesting observation.

This work reports for the first time that the endogenous lipid mediator LXA_4, not only plays a role in regenerating an ASL but also plays a significant role in protecting against *P. aeruginosa* invading and transmigrating across the epithelial layer by increasing ZO-1 protein expression, resulting in subsequent higher TJ support and function as which is demonstrated in Figure 6.15.

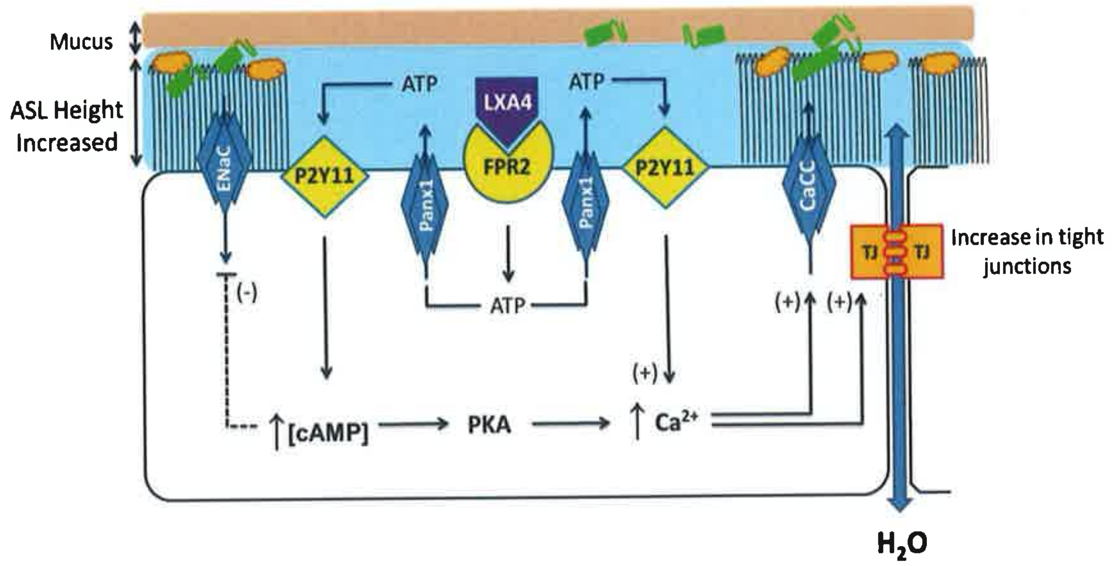


Figure 6.15: LXA_4 delays the invasion of bronchial epithelium cells by the pathogen *P. aeruginosa.* Cellular diagram showing the role that LXA_4 plays in protecting bronchial epithelial cells during infection. The epithelial barrier integrity is protected by an increase in ASL height induced from purinoreceptor (P2Y11) stimulation by ATP activating chloride secretion through a calcium activated chloride channel (CaCC) which in turn inhibits the sodium epithelial channel (ENaC) and by an increase in tight junction (TJ) formation at the apical membrane. These physiological effects induced by LXA_4 delay the invasion of bronchial epithelial cells by *P. aeruginosa.*

Chapter VII - General Discussion

7.1. General discussion

The work presented in this thesis demonstrates a novel role for LXA_4 to restore ion transport and ASL function in bronchial epitelium. Our studies indicate LXA_4 as a therapeutic option for CF lung disease. The binding of LXA_4 and its stable analogue TA39 to the ALX/FPR2 receptor induces an apical ATP release stimulating an increase in ASL height. The decrease in ASL height is a central mediator to the inflammation/infection cycle observed in the CF lung. Rehydration of the ASL is used as therapy by other agents within the lungs with effective results, however, LXA_4 as reported here not only acts as mediator of the resolution of inflammation, it also increases ASL height and tight junction formation. The delay in invasion of bronchial epithelium cells by the pathogen *P.aeruginosa* when epithelial cells were stimulated with LXA_4, suggests that LXA_4 may also be used as a therapy in parallel with antibiotics to decrease bacterial burden on the CF lung.

The mutation in the CFTR leading to inhibition of Cl^- transport results in an impaired ASL layer height in the lungs of patients with CF. This deficiency in ASL height leads to severe chronic airway disease featuring mucus obstruction, increase in bacterial burden and neutrophil-dominated airway inflammation. Bacterial infection and the exaggerated inflammatory response to persistent infection, facilitate tissue distraction and tissue remoulding leading to progressive pulmonary damage resulting in early morbidity and mortality for the CF patient. To increase the life expectancy of the CF patient, novel therapeutics are required that can increase ASL height and restrain the extreme reaction of the inflammatory system within the CF lung. LXA_4 and its stable analogues maybe regarded as one of these molecules by increasing ASL height, increasing tight junction formation and limiting the invasion of epithelial cells by the pathogen *P.aeruginosa* along with its native role as a lipid mediator in the resolution of inflammation (4).

The resolution of inflammation once defined as a passive process is now known to be tightly regulated by lipid mediators such as lipoxins, resolvins, protectins and maresins. The CF lung has been identified as being more pro-inflammatory than normal lung. It is not currently understood why the CF lung has this predetermined proinflammatory function (91, 167, 168). Previous reports have suggested that LXA_4 the pro-resolving endogenous lipid mediator is decreased in the lungs of CF patients (3). This decrease in LXA_4 could mediate the vicious proinflammatory cycle observed in the CF lung. The role of LXA_4 as a proresovling mediator is well documented. However, the role that LXA_4 plays in the lungs of CF patients is not clearly defined.

In normal airways CFTR acts as a cAMP-dependent Cl^- channel that works alongside a CaCC to secrete Cl^-, which is required for hydration of the airways. CFTR also functions as a regulator of ENaC that creates the limiting pathway for Na^+ absorption, which taken together play a key role in the regulation of ASL height. It has been previously shown that depletion in ASL height and volume impairs ciliary beat frequency, mucus transport, mucus stasis and adhesion of mucus to epithelial cells The defective mucocilicary clearance facilitates the bacterial adherence and colonisation of the lungs (47, 49, 53, 63, 169). LXA_4 as shown in Chapter III increases ASL height in non-CF cell lines, CF cell lines and primary CF bronchial epithelium cultures, which is mediated by ion transport, in particular stimulation of Cl^- secretion, that leads to an inhibition of sodium absorption, is the first of its kind to be reported. The fact that the ASL height increase stimulated by LXA_4 is observed not only in NuLi-1 cells (non-CF) but also in CuFi-1 cells (CF) and primary cultures of CF bronchial epithelial cells is coherent with the idea that the ASL height increase is stimulated via an alternative chloride pathway (distinct from CFTR) which is crucial for CF suffers.

The duration of the ASL height increase over 24 h induced by LXA_4 was observed in CF bronchial epithelium. Although the ALI method of HBE cell culture is well regarded as physiologically relevant, the lack of peripheral blood mononuclear cells, enzymes and fluid normally associated within the lungs cannot be accounted for and therefore limits the ability of this airway model. LXA_4 is degraded and inactivated rapidly within the body (106, 127). Therefore a stable analogue of LXA_4 would be advantages in in this setting. TA39 the stable analogue of LXA_4, mimicked the effect of the ASL height increase induced by LXA_4. It would be interesting to investigate further, TA39's physiological effects on bronchial epithelium such as migration, proliferation, Cl^- secretion and tight junction formation which has already been reported as physiological responses induced by LXA_4 on bronchial epithelium (120, 126, 129, 141).

The ASL height increase induced by LXA_4 and TA39 was inhibited by the ALX/FPR2 receptor antagonist Boc-2. This indicates that the ASL height response to LXA_4 is mediated by the ALX/FPR2 receptor which is consistent with other reports of receptor activation (128). In contrast, a study conducted by *Hansen et al* 2013, has stated that LXA_4 physiological response in epithelium is not mediated through the ALX/FPR2 receptor. This group found LXA_4 did not increase Ca^{2+} nor did it increase cAMP levels in transfected CHOand HEK293 cells (170). Christophe *et al 2002* also showed that HL60 cells transfected with ALX/FPR2 did not induce a rise in Ca^{2+} (171). It may be possible that these cell types do not express the specific G proteins that regulate the ALX/FPR2 receptor. Another question needs to be addressed in regards to the expression of the required puriniergic receptors located on the cell membrane that induces the increase in Ca^{2+} and cAMP levels by downstream of activation of the P2Y receptors as reported in this body of work.

There have been previous reports suggesting that nucleotides in particular ATP and its metabolite ADP play a major role in regulating ASL height. The ATP released from the NuLi-1, CuFi-1 and primary CF bronchial epithelial cells when stimulated with LXA_4 was in the µM range. Reports have suggested that ATP is steadily released in the airways at resting conditions but increase in ATP to the levels of nM are required for the activation of P2Y receptor The apically expressed

P2Y receptors can increase chloride secretion via IP3, increase intracellular Ca^{2+} and limit the rate of sodium absorption. P2Y2 receptor has been indicated as the main receptor responsible for the molecular effects of ATP on ASL height (48, 56, 57, 59, 62, 172). However, this thesis suggests for the first time that P2Y11 the receptor has greater responsibility in regulating ASL height increase induced by ATP. The increase and rapid relocation of the P2Y11 receptor to the apical membrane in bronchial epithelium would seem to confirm this assumption, which was explainedin Chapter IV. The P2Y2 receptor may still have a role to play in ASL height increase in bronchial epithelium as UTP along with ATP are P2Y2 receptor agonists (173). The fact that there is no specific chemical inhibitor of the P2Y2 receptor, silencing the P2Y2/P2Y11 receptors by siRNA could determine if P2Y2 has a role to play in the ASL height induced by LXA_4 and would merit further investigations.

Panx1 channels play a major role in the release of ATP. This is the first report to show that the lipid mediator LXA_4, produces an ATP release, from as yet an unknown source, in bronchial epithelium which is released apically by Panx1 channels. Panx1 channels have been shown to form protein-protein association with the purinergic P2X7 receptor. Activation of this receptor with ATP opens a cationic channel within milliseconds followed by an opening of a large pore permeable to molecules 900 Da in size. Mechanical and osmotic stimuli and changes to intracellular calcium have also been suggested to open the Panx1 channel releasing ATP in the absence of P2X7 receptors. The conformation changes within the cell i.e. increase in ZO-1 and the rapid localisation of receptors to the apical membrane suggest that the ATP release by Panx1 channels may be induced by a conformational change within the cell structure. The activity of Panx1 channels can be blocked with a number of inhibitors, that are thought to be specific for other proteins such as;several transport inhibitors, chloride channel blockers, inhibitors of P2X7 receptor and inflammasome inhibitors (174-176). The wide range of inhibition may suggest that Panx1 may possess a wider range of physiological functions or they could share the same structural protein domains with other proteins that have not yet been identified. It has not been reported that P2Y or ALX/FPR2 receptors may have protein-protein interactions or share some sort of structural domain with Panx1 channels.

The role of ATP in regulating ASL height, which has been reported in this body of work, is consistent with studies produced and reviewed by Boucher and Tarran (48, 50). ATP also plays a critical role in the effective ciliary beat frequency (177). Therefore the increase in ATP released from bronchial epithelium cells when stimulated with LXA_4 may also affect the frequency and effective beating of cilia. LXA_4 stimulation of cilia beat frequency in addition to an increase in ASL height would increase the efficiency of the defective mucociliary clearance in CF.

Polarised epithelial cells are surrounded by two morphologically well-defined intercellular junctions; the AJs, which mediate cell-cell adhesion and signalling pathways and the second being TJ which regulate the movement of ions, macromolecules and immune cells through the paracelluair space (152). The scaffolding protein ZO-1 is required for the formation of TJs. The depletion of ZO-1 results in an increase in cell permeability, a delay in the formation of the AJs results in cells that are less uniform in their epithelial packing with an increase in height observed (89, 152, 178, 179). The increase in ZO-1 protein in bronchial epithelial cells upon stimulation with LXA_4 provides further evidence for the protective role that LXA_4 plays within the lung. The rapid transolacation of ZO-1 to the apical barrier and increase in protein expression upon stimulation was observed in CF bronchial epithelium. It has previously been reported that CF cells display a reduction in basal repair levels. *Buchanan et al* 2013, has recently shown that LXA_4 increases the levels of repair in non-CF and CF bronchial epithelium (141). The reduction of LXA_4 in the lungs of CF patients may be a contributing factor to this delay. It would be interesting to note if different members of the TJ and AJ complex show an increase in expression upon stimulation with LXA_4. Depletion of both the ZO-1 and ZO-2 proteins from epithelial cells increases further and disruption of the transmembrane proteins at the tight junction and causes a dramatic alteration of TJ barrier compared to the loss of just one ZO protein (179). Other domains within the TJs like the claudins, JAMS and tight junction–associated MARVEL protein (TAMPS) may also be regulated by the addition of LXA_4, resulting in an increase in cell support and function.

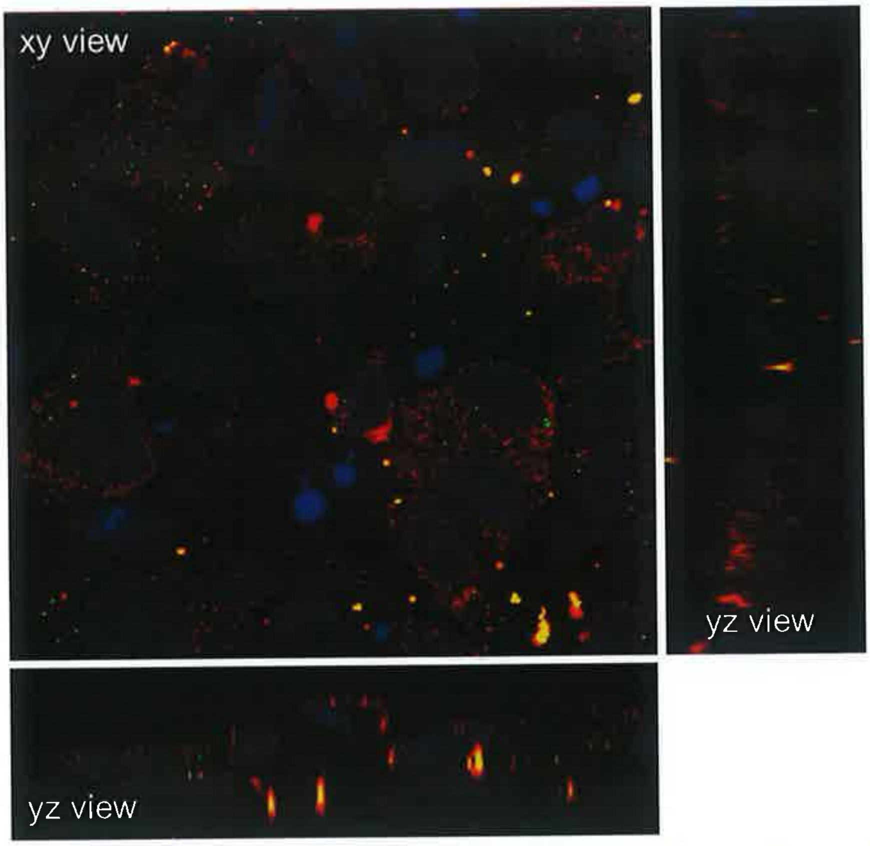
The epithelial dysfunction and pathogen sensing within the CF airway lead to recruitment of leukocytes via circulation into the airways. A hallmark of CF lung disease is the inflammation dominated by neutrophils. Neutrophils travel to the circulatory system daily; upon infection they rapidly transmigrate to the site of inflammation where they sense PAMPs /DAMPs (91). The overwhelming number of neutrophils in CF lungs can cause significant lung tissue damage when they accumulate over long periods of time and liberate their toxic granule contents in an uncontrolled fashion (91) . LXA_4 may ease the effect of this persist neutrophil migration and the uncontrolled release of their toxic material. In response to tissue injury or infection, arachidonic acid, the precursor of LXA_4, produces proinflammatory LTB_4 that leads to neutrophil recruitment and inflammation. LXA_4 displays potent anti-inflammatory actions including the reduction of neutrophil respiratory burst. Interaction of LXA_4 with its receptor ALX/FPR2 inhibits the initiated respiratory burst, chemotaxis, adhesion and transmigration induced by LTB_4 (118, 180). The restoration of TJs upon stimulation of LXA_4 closes off the paracellualr pathway. This closing of the paracelluar pathway maybe another physiological mechanism induced by LXA_4 leading to decreasing numbers of transmigrating neutrophils primed for activation.

The decrease in ZO-1 protein in bronchial epithelium upon contact with *P. aeruginosa* is directly proportional to the increase in *P. aeruginosa* invading bronchial epithelial cells as reported in Chapter V. This result demonstrates another physiological protective mechanism of LXA_4 on bronchial epithelium. The stimulation of ZO-1 protein expression by LXA_4 exposure after infection with *P. aeruginosa* is crucial for the cell to function under the threat of infection as ZO-1 must localise to the junction in order to rescue permeability and TJ formation (179). If ZO-1 is removed or lost from the cell, then TJs ability to regulate AJs is also lost. This will result in the loss of cell-cell communication (89). This loss will lead to a 'leakier' paracellular route which favours invasion of pathogens resulting in further damage to the protective epithelial layer. Results from our lab have shown that LXA_4 increases epithelial repair after injury and stimulates cell proliferation and migration by activation of K_{ATP} potassium channel (141). It would be appealing to see if TJ proteins are manipulated in this process of migration when stimulated with epithelial cells and if there is an increase observed in TJs proteins that have been mechanically injured and treated with LXA_4 compared to non-stimulated epithelial cells.

7.2. Future directions

Mucins, glycoproteins that are present between epithelial layers and the extracellular environments, are required in the airways for the effective sputum clearance along with protecting the airways from pathogens and dehydration. There are conflicting reports in the literature on the secretion of mucins within the CF lung. Reports by Rubin *et al* (181) suggest secretion of mucins is at normal levels in patients with CF but Hanke *et al* (182) suggest that MUC5AC and MUC5BC mucins are increased in the lungs of CF patients during exacerbations while Boucher *et al* indicates that mucins become dehydrated which changes the conformation of the mucins, resulting in extracellular formation of interchain bonds among airway mucins, making them increasingly difficult to remove from the CF lung (131, 181, 182). It has not yet been reported if LXA_4 may affect the levels of secretion and conformation of mucins and this would be an important area for future investigations. Confocal microscopy and protein determination of mucins can be used for the evaluation viscosity, volume and release of mucins when bronchial epithelium cells are stimulated with LXA_4. In collaboration with M. Clynes of University College Dublin, preliminary data were obtained showing that *P. aeruginosa* preferentially adheres to MUC5AC on CF bronchial epithelium. Could the rehydration of mucus by the increase in ASL height induced by LXA_4 reduce the number of bacteria and mucins in contact with epithelial cells? Investigation of this possibility would provide an interesting insight into the role of mucins and LXA_4 in CF infections.

The ability of *P. aeruginosa* to invade epithelial cells has been controversial. There are a now growing number of studies that support the ability of *P. aeruginosa* to invade epithelial cells (142, 146, 147). Although the work presented here has shown that LXA_4 decreases the number of *P. aeruginosa* invading epithelial cells, it is still not clear as to what paramater is driving the invasion i.e. are the bacteria unleashing there potent arsenal of biological weapons to invade epithelial cells or are the epithelial cells using phagosomes to destroy bacteria within lysosomes. Phagosome formations are crucial for microbial killing and antigen presentation to leukocytes. Rab proteins coordinate the consecutive stages of transport of vesicle formation and motility within the cell with Rab5 protein required for successful phagosomal formation that must precede Rab7 which is required for fusion of phagosomes with late endocytic compartments (183). Future investigations could focus on this phagosomal pathway, if LXA_4 plays a significant role in the recruitment of Rab proteins to the phaogsome and if there is a defect in the killing within the phagosome. As shown in Figure 7.1, it is possible to image the invading bacteria and Rab proteins. Reports have suggested that lysosomes within CF cells do not have the ability to destroy microorganisms because of the defunctional CFTR resulting in a defective acidification of the lysosome (184). Bacteria could use this defective lysosome to evade the full effects of host immunity and multiply within the cell. In our studies the increase in the numbers of viable bacteria that had invaded epithelial cells in non-stimulated cells warrants further investigations, for example, lipoxin effects on the pH within epithelial cells and phagosome.



Blue – Nucleus Red – Rab5 Green – *P. aeruginosa* Yellow – Co-localisation

Figure 7.1: Phagosomal proteins are co-localised with *P. aeruginosa* in NuLi-1 cells. NuLi-1 cells infected with *P. aeruginosa* were imaged by immunofluorescence and confoucal microscopy 24 h post-inoculation. Bacteria (green) that have invaded the epithelial cells are co-localised (yellow) with the Rab5 protein (red) which is associated with the early phagosome. This preliminary result indicates that not only does *P.aeruginosa* invade bronchial epithelium cells but does so with the use of the phagosomal pathway.

The infection model devised in this study (addition of bacteria to fully differentiated bronchial epithelium) only centres on the physiological effects of epithelial cells and bacteria. There could be arguments made of the physiological relevance of this infection model. To advance the reconstituted epithelium airway infection model the addition of neutrophils and leukocytes to the airway model is proposed. The addition of these cells to the infection model would increase the molecular and physiological understanding of infection with CF without the need of expansive animal models while chemokine, cytokine, proinflammatry and pro-resolution mediators may all be measured in real time at the site of infection. The antimicrobial peptide LL-37 is realised from both neutrophils and epithelial cells (162). There was no difference in the amount of LL-37 released from epithelial cells reported in our infection model. It would be appealing to observe the amount of LL-37 released from neutrophils and epithelial cells in a more complex infection model. Indeed this could also include various other antimicrobial peptides found not only released from epithelial cells but also from neutrophils such as α-defensins, β-defensins, calprotectins and lactoferins. With the addition of LXA_4 to this more complex model concentration of microbial peptides released could be measured along with the numbers of migrating neutrophils. It is also reported that neutrophils change their phenotype to produce different lipid mediators depending on the cells and substrates present. The advancement of the airway culture model will give a greater insight as to when these switches may occur and if the switching can be enhanced upon the addition of LXA_4.

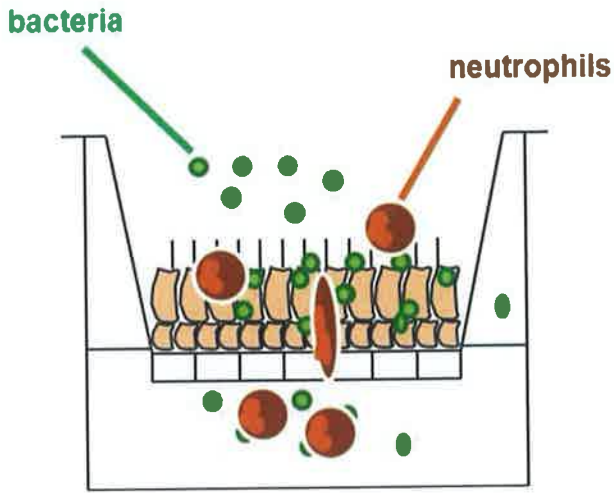


Figure 7.2: Proposed infection model

To further understand the effect of LXA_4 on bronchial epithelium a more complex infection model is proposed. With the addition of neutrophils or other leucocytes to the airway model will ensure more physiological response to infection, measuring of antimicrobial peptides, chemokines, cytokines and pro-resolution mediators can all be measured in real time. This model could also allow for the quantifying or visualisation (by live cell imaging) of neutrophil migration and the inhibitory effect LXA_4 has on this process.

The role of specialised proresolution lipid mediators has been clearly described. Lipoxin, resovlins, protectants and maresins (167, 168, 185) all play fundamental roles in the complete resolution of inflammation. Karp et al has shown that a reduction in LXA_4 within the CF lung attenuates disease severity (3). The addition of antibiotics with LXA_4 to the infection model used in this report may show encouraging results as the role of lipoxins, resolvins, protectins and maresins are not immunosuppressive but act to support the resolution of inflammation and activate homeostasis of tissue. The addition of these proresolving mediators along with antibiotic treatment will not overwhelm the immune system but will act as an aid in the rapid resolution of infection and inflammation and further investigation of this novel treatment is required. However, further questions may need to be addressed from this work; When are each of the proresolving mediators activated and within what time frame does the class switching occur from lipoxin-resolvins-protectins-maresins? The answers to these questions my lay in the fate of the CF pig which initially developed in the University of Iowa and now further investigated in Europe (186, 187). The CF pig was developed to research the pathogenesis of the CF lung disease and the future testing of CF therapies. The CF pig develops the major manifestations of the human CF lung disease (188). Samples of fluid or lung tissue from the CF pig could be examined at different stages of exacerbation. During these different stages measuring the amounts of the proresolving mediators should allow for a greater knowledge of when LXA_4 should be used as an adjunct therapy along with other family members of the resolving family. The decision as or when to add LXA_4 could be made to mimic the increase in proresolving mediators of that observed in native conditions for e.g. A complex study could see the addition of *P. aeruginosa* to the lung of the CF pig. To clear the infection treatment with antibiotics along with the administering of proresolving mediators (such as LXA_4 and RvD1) could be administered at same time or in latter stages of antibiotic treatment depending on the results observed in the CF pig. This treatment could help alleviate infection/inflammation and exacerbations associated with CF lung disease.

7.3 Concluding remarks

This thesis has demonstrated that the lipid mediator LXA_4, has a novel role in the regulation of ASL height in CF bronchial cells by the alteration of Cl^- secretion by downstream activation of the purionreceptor pathway. This work reports for the first time that the activation of the ALX/FPR2 receptor by its agonist LXA_4 rapidly increases the P2Y11 receptor expression at apical membrane of bronchial epithelia. This increase in receptor expression may enhance the ASL response to lipoxin by increasing intracellular Ca^{2+} and cAMP leading to a sustained ASL height increase for up to 24h in CF airway. Furthermore, this body of work has also revealed for the first time that LXA_4 increases the tight junction protein ZO-1 in CF bronchial epithelium cells. The increase in ZO-1 had a protective role against the invasion of bacteria across the epithelial barrier. Taken together this thesis provides evidence that LXA_4 may not only play a role in resolving inflammation but also act as a potential therapeutic avenue leading to the resolution of CF lung disease.

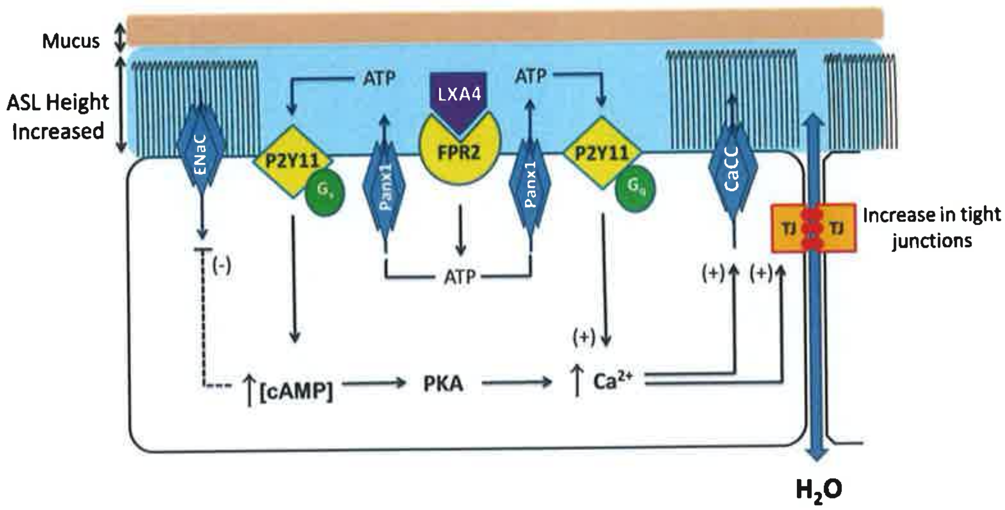


Figure 7.3: Physiological effects of LXA_4 on CF bronchial epithelium. Stimulation of the FPR2 receptor by LXA_4 induces an apical ATP release activating a purinoreceptor pathway, more specifically the P2Y11 receptor. Unique amongst its family of receptors P2Y11 is double linked to two G-proteins, G_s and G_q proteins that activate a intracellular Ca^{2+} release, leading to an increase Cl^- transport out of the cell by a calcium-activated chloride channels (CaCC). The activated Cl^- transport and inhibition of sodium absorption through an epithelial sodium channel (ENaC) restrores rehydration of the ASL. LXA_4 also protects epithelial barrier integrity by increasing ZO-1 protein synthesis, promoting tight junction (TJ) resistance and function. Taken together LXA_4 has potential as a new therapeutic route in the treatment of CF lung disease.

Appendix

Patient Form HCFBE14

Date : 23/02/11	Culture code : HCFBE14

Patient Number : PO37	Sample number : 1

Sample type

☒ Bronchial brushing ☐ Nasal Brushing ☐ Bronchial biopsy

Patient

☒ Male ☐ Female **Age: 3 yr**

Genotype: ☐ non-CF ☐ normal
☐ Asthma
☐ Dyskinesia
☐ Other: ____________

☒ **CF**

ΔF508/□F508: ☒
ΔF508/X: ☐ X :
Other : ☐
Unknown * : ☐ Genotyping date : 01/10/2007
Genotype :

Infection agents risk:
☒ Staphylococcus Aureus (2010) ☐ Pseudomonas Aeruginosa ☐
Aspergillus Fumigatus
☐ Mycobacterium Avium
☐ Other :

Candida 2010
Haemophilus influenzae 2010
Strep. pneumoniae

Actual patient treatment (Drugs):
☐ Antibiotherapy:
☐ Anti-inflammatory therapy:
☒ Other: Creon, Dalavit, VitE, VitK, NaCl 7% (hypertonic saline)

Patient Form HCFBE16

Date : 20/04/2011	Culture code : HCFBE16

Patient Number : PO43	Sample number : 1

Sample type

☒ **Bronchial brushing** ☐ **Nasal Brushing** ☐ **Bronchial biopsy**

Patient

☒ **Male** ☐ **Female** **Age: 3 yr**

Genotype: ☐ **non-CF** ☐ normal
☐ Asthma
☐ Dyskinesia
☐ Other: ____________

☒ **CF**

ΔF508/□F508: ☒
ΔF508/X: ☐ X :
Other : ☐
Unknown * : ☐

Genotyping date : 03/03/09
Genotype :

Infection agents risk:
☐ Staphylococcus Aureus ☐ Pseudomonas Aeruginosa ☐ Aspergillus Fumigatus
☐ Mycobacterium Avium
☐ Other :

Actual patient treatment (Drugs):
☐ Antibiotherapy:
☐ Anti-inflammatory therapy:
☐ Other:

Patient Form for HCFBE20

Date : 18/05/11	Culture code : HCFBE20

Patient Number : P048	Sample number : 1

Sample type

☒ **Bronchial brushing** ☐ **Nasal Brushing** ☐ **Bronchial biopsy**

Patient

☒ **Male** ☐ **Female** **Age: 3 yr**

Genotype: ☐ **non-CF** ☐ normal
☐ Asthma
☐ Dyskinesia
☐ Other: ____________

☒ **CF**

ΔF508/□F508: ☒
ΔF508/X: ☐ X :
Other : ☐
Unknown * : ☐

Genotyping date : 7/05/2008
Genotype :

Infection agents risk:
☐ Staphylococcus Aureus ☐ Pseudomonas Aeruginosa ☐ Aspergillus Fumigatus
☐ Mycobacterium Avium
☒ Other :

Throat swab- 12/4/2011 Haemophillus influenza
Throat swab – 15/2/2011 Candida species
Throat swab – 16/12/2010 Step. pneumoniae

Actual patient treatment (Drugs):
☐ Antibiotherapy:
☐ Anti-inflammatory therapy:
☒ Other:

Patient Form for HCFBE68

Date : 18/01/2012	Culture code : HCFBE68
Patient Number : P068	Sample number : 1

Sample type

☒ **Bronchial brushing** ☐ **Nasal Brushing** ☐ **Bronchial biopsy**

Patient

☐ **Male** ☒ **Female** **Age: 4**

Genotype: ☐ **non-CF** ☐ normal
☐ Asthma
☐ Dyskinesia
☐ Other: ____________

☒ **CF**

ΔF508/□F508: ☒
ΔF508/X: ☐ X :
Other : ☐
Unknown * : ☐

Genotyping date : 14/12/2007
Genotype :

Infection agents risk:
☒ Staphylococcus Aureus ☐ Pseudomonas Aeruginosa ☐ Aspergillus Fumigatus
☐ Mycobacterium Avium
☐ Other :

Candida species –from throat swab

Actual patient treatment (Drugs):
☐ Antibiotherapy:
☐ Anti-inflammatory therapy:
☐ Other

Patient Form for HCFBE69

Date : 18/01/2012	**Culture code : HCFBE69**

Patient Number : P069	Sample number : 1

Sample type

☒ **Bronchial brushing** ☐ **Nasal Brushing** ☐ **Bronchial biopsy**

Patient

☐ **Male** ☒ **Female** **Age: 4**

Genotype: ☐ **non-CF**
- ☐ normal
- ☐ Asthma
- ☐ Dyskinesia
- ☐ Other: ____________

☒ **CF**

ΔF508/ΔF508: ☐
ΔF508/X: ☒ X : 1766+1G>A
Other : ☐
Unknown * : ☐

Genotyping date :
Genotype :

Infection agents risk:
- ☐ Staphylococcus Aureus ☐ Pseudomonas Aeruginosa ☐ Aspergillus Fumigatus
- ☐ Mycobacterium Avium
- ☐ Other :

Haemophilus

Actual patient treatment (Drugs):
- ☐ Antibiotherapy:
- ☐ Anti-inflammatory therapy:
- ☐ Other

Patient Form for HCFBE76

Date : 14/03/2012	Culture code : HCFBE76

Patient Number : P076	Sample number : 1

Sample type

☒ Bronchial brushing ☐ Nasal Brushing ☐ Bronchial biopsy

Patient

☒ Male ☐ Female **Age: 4**

Genotype: ☐ non-CF
☐ normal
☐ Asthma
☐ Dyskinesia
☐ Other: ______________

☒ CF

ΔF508/□F508: ☒
ΔF508/X: ☐ X :
Other : ☐
Unknown * : ☐

Genotyping date :
Genotype :

Infection agents risk:
☐ Staphylococcus Aureus ☐ Pseudomonas Aeruginosa ☐ Aspergillus Fumigatus
☐ Mycobacterium Avium
☐ Other :

Normal flora

Actual patient treatment (Drugs):
☐ Antibiotherapy:
☐ Anti-inflammatory therapy:
☒ Other : creon, vitamin K

Patient Form for HCFBE90

Date : 09/05/2012	Culture code : HCFBE90 P0

Patient Number : P090	Sample number : 1

Sample type

☒ **Bronchial brushing** ☐ **Nasal Brushing** ☐ **Bronchial biopsy**

Patient

☒ **Male** ☐ **Female** **Age:**

Genotype: ☐ **non-CF** ☐ normal
☐ Asthma
☐ Dyskinesia
☐ Other: ____________

☒ **CF**

ΔF508/□F508: ☒
ΔF508/X: ☐ X :
Other : ☐
Unknown * : ☐

Genotyping date : 03/03/2009
Genotype :

Infection agents risk:
☐ Staphylococcus Aureus ☐ Pseudomonas Aeruginosa ☐ Aspergillus Fumigatus
☐ Mycobacterium Avium
☐ Other :

Actual patient treatment (Drugs):
☐ Antibiotherapy:
☐ Anti-inflammatory therapy:
☐ Other :

Patient Form for HCFBE107

Date : 26/09/2012	Culture code : HCFBE 107 P0

Patient Number : P107	Sample number :

Sample type

☒ **Bronchial brushing** ☐ **Nasal Brushing** ☐ **Bronchial biopsy**

Patient

☒ **Male** ☐ **Female** **Age: 4yr**

Genotype: ☐ **non-CF** ☐ normal
☐ Asthma
☐ Dyskinesia
☐ Other: ____________

☒ **CF**

ΔF508/□F508: ☐
ΔF508/X: ☐ X :
Other : ☐
Unknown * : ☐

Genotyping date : 03/03/2009
Genotype :

Infection agents risk:
☐ Staphylococcus Aureus ☐ Pseudomonas Aeruginosa ☐ Aspergillus Fumigatus
☐ Mycobacterium Avium
☐ Other :

Normal flora,

Actual patient treatment (Drugs):
☐ Antibiotherapy:
☐ Anti-inflammatory therapy:
☐ Other :

Patient Form for SC004CB3

Date : 23/01/2013	Culture code : SC004CB3
Patient Number : SC004CB3	Sample number : 1

Sample type

☒ **Bronchial brushing** ☐ **Nasal Brushing** ☐ **Bronchial biopsy**

Patient

☒ **Male** ☐ **Female** **Age: 4yr**

Genotype: ☐ **non-CF** ☐ normal
☐ Asthma
☐ Dyskinesia
☐ Other: ________________

☒ **CF**

ΔF508/□F508: ☐
ΔF508/X: ☒ X : 2184dela
Other : ☐
Unknown * : ☐ Genotyping date :
Genotype :

Infection agents risk:
☐ Staphylococcus Aureus ☐ Pseudomonas Aeruginosa ☐ Aspergillus Fumigatus
☐ Mycobacterium Avium
☐ Other :

Actual patient treatment (Drugs):
☐ Antibiotherapy:
☐ Anti-inflammatory therapy:
☐ Other :

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